METABOLIC SYNDROME, THE LEPTIN GENE AND KIDNEY DISEASE IN NON-DIABETIC BLACK SOUTH AFRICANS.

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"Let me have men about me that are fat, sleek-headed men, and such as sleep o'nights: Yond Cassius has a lean and hungry look; he thinks too much; such men are dangerous"

From Julius Caesar (Act I, Scene II)

William Shakespeare

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DECLARATION

I, Ikechi Gareth Okpechi, hereby declare that the research described herein was performed by me with the assistance as indicated in the acknowledgements. The dissertation was written by me and reviewed by my supervisor. Neither the whole thesis nor any part thereof has been, is being or will be submitted by me for any other degree at this or any other University.

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Ikechi Gareth Okpechi Date

DEDICATION

In memory of my late father and friend – Chike Okpechi.

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ABSTRACT

Obesity is a worldwide problem and is a factor in the pathogenesis of the metabolic syndrome and kidney disease through the development of obesity-related hypertension and neuro-hormonal mechanisms that include the action of leptin.

As there appear to be no focussed studies that have looked at the association of the LEP gene with kidney disease phenotypes or cardiovascular disease markers like hypertension, the metabolic syndrome and obesity, and especially so in native black Africans, this study sought to establish an association between the obesity gene (LEP) and kidney disease phenotypes (independent of diabetes and hypertension) in a homogenous black African population. This study was of cross – sectional design and was carried out in the hypertension clinic of a native black South African population.

The study was also designed to identify the prevalence of genotypes of the LEP gene in black South Africans, the relationship between the LEP gene, obesity, hypertension, the Metabolic Syndrome, markers of kidney disease and serum leptin concentration, the effect of the metabolic syndrome on kidney disease phenotypes in this population and to identify candidate genes for the metabolic syndrome through a bioinformatics approach.

We not only confirmed results from other population in which kidney disease has been shown to be associated with the metabolic syndrome but also found significant inverse correlation between leptin and body mass index with glomerular filtration rate, making a strong case for obesity as a significant contributor to kidney disease.

We also report, for the first time, an association between the A19G genotypes of the LEP gene with kidney disease in non-diabetic hypertensives and normotensive subjects. Individuals with the AA genotype of this polymorphism had significantly higher albumin-to-creatinine ratio compared to subjects with the AG or GG genotypes. We also identified two haplotypes of the leptin gene with effects on kidney disease markers; one of the haplotypes (GCAT) appeared to have a protective effect on markers of kidney disease while the second haplotype (GAAC) was observed to have an effect that increased urine ACR, possibly making it a risk haplotype for renal disease.

Although an association between the LEP gene and microalbuminuria was seen in this population, we found no association between the LEP gene and obesity and the metabolic syndrome, considered as important driving factors for kidney disease. Therefore, we used a bioinformatics approach to select 19 top-scoring candidate genes for the metabolic syndrome. The candidates selected are found to most commonly represent pathways involving metabolism of lipids and lipoproteins, as well as transmembrane signalling and subsequent activation of signal transduction pathways.

ABBREVIATIONS

ACR	Albumin-to-creatinine ratio
ATPIII	Adult treatment panel (III)
BMI	Body mass index
CKD	Chronic Kidney disease
CVD	Cardiovascular disease
DAVID	Database for Annotation, Visualization and Integrated Discovery
DBP	Diastolic blood pressure
DGP	Disease gene prediction
ESRD	End-stage renal disease
FBG	Fasting blood glucose
FFA	Free fatty acids
G2D	Genes 2 Disease
GBD	Global burden of disease
GFR	Glomerular filtration rate
GO	Gene Ontology
HDL	High density lipoprotein
HOMA	Homeostasis model assessment
LDL	Low density lipoprotein
LEP	Leptin (gene)
MAP	Mean arterial blood pressure
MS	Metabolic syndrome
NCD	Non-communicable disease
NHANES	National health and nutrition education survey
RAAS	Renin-angiotensin-aldosterone system
RRT	Renal replacement programme
SADHS	South African demographic and health survey
SADTR	South Africa dialysis and transplant registry
SBP	Systolic blood pressure
SNP	Single nucleotide polymorphism
T2DM	Type 2 diabetes mellitus
TG	Triglycerides
UTR	Untranslated region
WC	Waist circumference
WHO	World health organisation
WHR	Waist-to-hip ratio

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure 9.2.2 Potential binding interactions amongst candidate gene products, showing downstream signalling pathways activated.

CHAPTER 1

INTRODUCTION

1.1 NON-COMMUNICABLE DISEASES (NCDs)

There is an increasing tendency in today's world for the integration of ideas, people (culturally and politically), commerce, financial capital and technology - this is called globalisation and may have both beneficial and harmful effects on the health of populations (Woodward *et al* 2001). The effect of globalisation on health has for the most part been directed towards control of communicable diseases, provision of affordable medicines, and changes required in international trade to improve access to healthcare. By contrast, the growing burden of non-communicable diseases (NCDs) (such as kidney disease, heart disease, obesity, diabetes and stroke) has been neglected, especially in the developing countries of the world (Beaglehole and Yach 2003).

Non-communicable diseases are the largest cause of death in the world, led by cardiovascular disease (CVD) (17 million deaths in 2002, mainly from ischaemic heart disease and stroke) followed by cancer (7 million deaths), chronic lung diseases (4 million), and diabetes mellitus (almost 1 million) (WHO 2003). Moreover, the World Health Organisation (WHO) have declared that "obesity has reached epidemic proportions globally" and that "more than 1 billion adults are overweight and at least 300 million of them clinically obese" with obesity being a major contributor to the global burden of chronic disease and disability (WHO 2000).

In several developing countries there has been a rapid increase of chronic disease risk and mortality profiles (WHO 2002). For instance, in China, death rates from circulatory disease increased between 200% and 300% in those aged 35 through 44 years between 1986 and 1999, and by more than 100% in those aged 45 through 54 years (Yach *et al* 2004).

Although communicable diseases such as malaria, tuberculosis, HIV/AIDS are very common in developing countries, they are, in some cases, gradually becoming displaced by chronic NCDs. Thus, many developing countries are starting to experience a polarized and protracted double burden of disease (Popkin 2004; Frenk *et al* 1989). For example, India, with the highest number of diabetics in the world is projected to have an annual death from coronary heart disease of about 2 million by the year 2010 (Basnayar *et al* 2004). In addition, India still needs to be seriously concerned about the 2.5 million children who die from infectious diseases every year. In South Africa, infectious diseases such as HIV/AIDS and tuberculosis account for 28% of years of lives lost while chronic diseases account for 25% (Steyn *et al*., 2003).

Global projections of mortality and burden of disease from 2002 to 2030 have shown a dramatic shift in distribution of deaths from younger to older ages and from communicable, maternal, perinatal and nutritional causes to NCD causes. The proportion of deaths due to non-communicable causes is projected to increase from 59% in 2002 to 69% in 2030 with ischaemic heart disease as one of the top three disease burdens for that year (Mathers *et al* 2006).

In all countries, the increased burden of chronic NCDs has led to growing economic costs with increasing proof of this increased burden emerging for CVD, diabetes, and obesity (Thompson *et al* 2001). Many developed nations have focused considerable efforts on addressing the burden of chronic diseases. However, the rising burden of chronic NCDs on developing countries has received very little attention (Beaglehole and Yach 2003).

1.2 NON-COMMUNICABLE DISEASES IN AFRICA

In 1990 the Global Burden of Disease (GBD) study suggested that NCDs diseases accounted for only 14% of the total burden in sub-Saharan Africa (SSA) but the estimates also suggested that in absolute terms the probability of death from NCDs is higher in SSA than in developed nations (Unwin *et al* 2001). Due to the lack of reliable data from SSA, these estimates were heavily based on assumptions and extrapolations.

Tanzania, one of Africa's poorest countries has provided real data that can be used in support of these assumptions. Studies from this country (at least up until the year 2000) have shown that the probabilities of dying from a non-communicable cause are indeed higher than that from a communicable cause (Setel *et al* 2000, Kitange 1996). In addition, these studies show that among adults in Tanzania, the age-specific death rates from non-communicable diseases are substantially higher in all age groups when compared with developed countries.

The projections for SSA from the GBD study suggest that by the year 2020, the proportion of NCD will increase to somewhere between 26% and 34% and among adults aged 15 – 59 years to between 37% and 42% respectively (Unwin *et al* 2001). Despite these frightening figures, some have argued against an increased prioritization of NCDs in developing countries as it would work against the world's poorest 20%, many of who live in SSA. They

reason that in SSA, patients with NCDs only make a significant additional demand on an already depleted health care resources unable to cater for present problems of infectious diseases; hence that available scarce resources should be used to cater for patients with communicable diseases (Gwatkin *et al* 1999).

1.3 NCD: SOCIO-DEMOGRAPHIC FACTORS.

There are various factors that influence the global pattern of non-communicable diseases. Ethnicity is one such factor and encompasses both genetic and cultural differences (Anand *et al* 1999), and because individuals of different ethnic backgrounds tend to live in distinct regions and societies, variations in disease rates by ethnicity are also intertwined with geographic differences and may explain the variable impact of diseases in different populations. For instance, a specific ethnic group within a location may adopt a certain lifestyle, whereas the same ethnic group if in another location may adopt substantially different lifestyles.

Secondly, factors which promote selective survival of an individual in a population in response to the common environmental challenges experienced by that population also contribute to the variation of NCDs and especially CVDs. An example is the "thrifty gene hypothesis" that postulates that certain genes in humans have evolved to maximize metabolic efficiency, lipid storage and food searching behaviour, and that in times of abundance these genes predispose their carriers to diseases caused by excess nutritional intake, such as obesity (Hales *et al* 1992). This hypothesis has been used to explain the common occurrence of hypertension in some populations (Siani *et al* 2000).

Thirdly, the state of intrauterine, infant, and early childhood nutrition may later bring about population differences in CVDs (Barker 1994). For example, adverse foetal development due to poor maternal nutrition may confer a selective survival advantage to the foetus by programming it for reduced insulin sensitivity. However, as the child is exposed to excess nutrition in later years, such programming could lead to high blood pressure, glucose intolerance, and dyslipidemia.

Fourthly, differences in the epidemiologic transition of a country and exposure to specific associated environmental factors related to CVD risk may explain discrepancies in the risk of incident CVD within populations. This may provide the basis for differences in individual susceptibility to a particular disease in a common and relatively homogenous environment. The Ni-Hon-San study of Japanese migrants (Kagan *et al* 1974) provides a good example of how differences in demographic profiles, environmental factors, early childhood programming influences as well as differences in gene frequency or expression can contribute to variations in CVD between different populations. This study revealed how blood cholesterol levels and coronary heart disease rates rose from relatively low levels among those in Japan, to intermediate levels in Japanese migrants in Honolulu, and to high levels in those in San Francisco.

Finally, the rate of migration to western environments (often arising from urbanisation and related to epidemiologic transition) has been increasing globally, from 36.6% of the world population living in urban areas in 1970, to 44.8% in 1994. This proportion is projected to increase to 61.1% by 2025 (Chockalingam *et al* 1999). In a review of data on selected risk

factors related to the emergence of NCDs in South Africa Bourne *et al* (2002) described shifts in dietary intake, which from a health perspective was of a less prudent pattern, taking place with increasing momentum in urbanised black South Africans. This review also brought to light the report of the South African Demographic and Health Survey (SADHS) in which the increasing prevalence of hypertension, diabetes and especially obesity among black South Africans was described and related to dietary transition and urbanisation (Steyn *et al* 2003).

Therefore, the disparity in CVD rates observed in different parts of the world also mirror environmental changes usually arising from increasing urbanization with an associated dietary transition and a range of other influences occurring from early childhood to adulthood. Subsequent chapters will now attempt to trace the development of CVDs, factors which propel the increasing global incidence and prevalence of CVDs and particularly how all these may affect the kidneys.

CHAPTER 2

OBESITY

2.1 BACKGROUND

About 1 billion people in the world are overweight or obese, compared to 850 million who are underweight (WHO 2000). In the US, the National Health and Nutrition Examination Surveys (NHANES) show that the prevalence of obesity has risen across all age and ethnic groups substantially between 1976 and 1994, going from 14.5% to 22.5% (Flegal *et al* 1998).

Also, data from the adult health section of the 1998 SADHS had shown that the malnutrition pattern seen in adult South African population is one of predominantly over-nutrition rather than under-nutrition with a concomitant high prevalence of obesity, especially among black South Africans (Puoane *et al* 2002).

Obesity and overweight are the propellers of the global increase in type 2 diabetes, and both conditions have, during the past decade, joined underweight, malnutrition, and infectious diseases as the major health problems developing countries need to contend with (Haslam *et al* 2005). Approximately 60% of all cases of diabetes can be directly attributed to weight gain (James *et al* 2003). The rising incidence of concomitant obesity and type 2 diabetes, which was once considered a disease of adulthood, is increasingly being diagnosed in paediatric populations (ADA 2000).

The aim of this chapter is the consideration of the risks and complications associated with obesity. These metabolic disorders are discussed in the light of their relationship with CVDs. This chapter is an introduction to further discussion in the following chapters.

2.2 DEFINITION OF OBESITY

The world health organisation defines obesity based on the risks of increased mortality and morbidity (WHO 1997). A body mass index (BMI) below 18.5 kg/m^2 is defined as underweight; a BMI between 18.5 and 24.9 is normal weight. Pre-obese or overweight individuals are those with a BMI between 25 and 29.9 kg/m^2 and are at increased risk of morbidity and mortality from obesity complications. Obesity is conventionally defined as BMI $\geq$ 30 kg/m^2. (Table 2.1)

Sub-classification of obesity into class I, II, and III correspond to mild (BMI between 30 and 34.9 kg/m^2), moderate (BMI between 35 and 39.9 kg/m^2) and severe or morbid obesity (BMI $\geq$ 40 kg/m^2) respectively. Severely obese individuals are at higher risk of disease irrespective of the presence of other risk factors. Although the BMI is generally used to define obesity, other parameters such as waist circumference (WC) and waist-to-hip ratio (WHR) are also used and have ethnic specific cut-off points (Table 2.2).

Table 2.1: Definition and Classification of Obesity with associated Disease Risk.

			Disease risk relative to normal weight and waist circumference	
CLASS	**BMI** (kg/m^2)	**Obesity class**	Men ≤ 102 cm Women ≤ 88cm	Men ≥ 102 cm Women ≥ 88cm
Underweight	< 18.5		-	-
Normal	18.5-24.9		-	-
Overweight	25-29.9		Increased	High
Obesity	30-34.9	I	High	Very high
	35-39.9	II	Very high	Very high
Extreme obesity	≥ 40	III	Extremely high	Extremely high

Table 2.2: Ethnic specific Waist circumferences as recommended by the International Diabetes Federation.

Ethnic group	**Waist circumference.**
Europeans	Men: ≥ 94 cm Women: ≥ 80cm
South Asians	Men: ≥ 90 cm Women: ≥ 80cm
Chinese	Men: ≥ 90 cm Women: ≥ 80 cm
Japanese	Men: ≥ 85 cm Women: ≥ 90 cm
South and Central Americans	Use South Asian recommendations until more specific data are available
Sub-Saharan Africans	Use European recommendations until more specific data are available
Middle East (Arab) populations	Use European recommendations until more specific data are available

2.3 MEASUREMENT OF OBESITY

The most commonly used measurement for determining obesity is the BMI, which is calculated as the weight (kg)/height^2 (m^2) or as the weight (lb)/height^2 (in^2) x 703. The BMI provides a measurable estimate of body fat and is related to the risk of complications associated with obesity (Kushner *et al* 2003).

Anthropometry provides both absolute circumferential and skin fold measurements and derived estimates of body composition. Anthropometric measurements are made using simple devices such as the tape measure for circumferences and skinfold callipers for skinfolds (Table 2.3). Other methods, often used in research, include underwater weighing (hydro-densitometry) (Siri 1961), total body water estimation (Schoeller *et al* 1980), dual energy X-ray absorptiometry (DEXA) (Mazess *et al* 1984), bioimpedance analysis (Segal *et al* 1991) and imaging methods such as computed tomography (CT) and magnetic resonance imaging (MRI) (Lewis *et al* 1986). The later two methods can reliably differentiate between subcutaneous and visceral abdominal adipose tissue and are particularly useful for specific research studies.

Table 2.3: Anatomic location of skinfold and circumference measurement sites. (Blackburn *et al*, 1994)

Skinfold	Anatomic site
Bicep	A vertical fold taken anteriorly halfway between the acromian and olecranon process
Tricep	Avertical fold taken posteriorly to the bicep skinfold
Subscapular	A diagonal fold taken just below the inferior angle of the scapula
Suprailiac	A diagonal fold taken just above the iliac crest at the midaxillary line
Chest	A diagonal fold taken halfway between the armpit and the nipple for men and one-third the distance for women
Thigh	A vertical fold taken anteriorly halfway between the hip and the knee
Circumference	**Anatomic site**
Upper arm	Measured halfway between the acromian and olecranon processes
Chest	Measured at the nipple line in men; at the largest circumference above the breasts in women
Abdomen	The smallest circumference in the abdominal area
Waist	Measured at the level of the iliac crest
Hips	The largest circumference below the umbilicus
Buttocks	The largest circumference in the gluteal area
Thigh	Measured just below the gluteal fold
Calf	The largest circumference below the knee

2.4 OBESITY RISK FACTORS

Except in the rare monogenic forms, obesity tends to occur as a result of the interactions of several risk factors. These factors, working together with small contributions from each part eventually lead to the common obese phenotype.

2.4.1. GENETIC

The commonly observed coexistence of several obese members within a family suggests the involvement of genetic factors in obesity and the risk of excessive weight gain in children of some families with obese parents is increased two- to three-fold for moderate obesity and up to eight times for severe obesity (Bouchard 2001). This genetic influence affects African-American adolescents more so than white adolescents (Allison *et al* 1994). The genetics of obesity is to be further explored in chapter 6.

2.4.2. DIET

Diet plays possibly the biggest role in obesity. Generally, the three ways that relate diet to obesity are: (i) the amount of calories an individual consumes, (ii) changes of the dietary pattern of the general population and (iii) marketing strategies by food industries.

Firstly, cross-sectional studies have shown that obese subjects in general consume more calories than do normal-weight subjects (Astrup *et al* 1994). In a study of a rural South African population, comparison was carried out of nutrient intake between 1969 and 1989 (Walker *et al* 1992). Energy intake was reported to have risen from 7.2 to 7.8 MJ with a consequent increase of mean BMI by 5.4% in that population.

Secondly, dietary transitions also contribute significantly to obesity and have been reported from developing countries like those in Asia with ensuing increases in obesity prevalence in

such countries. In China for instance, changes in patterns of diet over the past few decades can be used to estimate the rising trend of obesity. Average daily energy intake per capita was 2060 Kcal in 1959 and had risen to 2485 Kcal in 1982. Also, while total energy consumption remained relatively stable between 1982 and 1992, the diet composition continued to change, with a larger proportion of energy provided by fat (18% of energy in 1982 compared with 22% in 1992) and lower consumption of carbohydrates (80% of energy in 1982 compared with 72% in 1992) (Zhou 1998). Concurrently, within a decade (1989 to 1997), the prevalence of obesity in urban Chinese children had risen from 1.5% to 12.6% (Luo *et al* 2002).

Thirdly, marketing of food has also greatly impacted on the changes observed in dietary patterns in different populations. Global marketing and the systematic moulding of taste by giant corporations has become a central feature of the globalization of the food industry (Barnett *et al* 1994). Big brand names like Coca Cola and Pepsi were recognized by 65% and 42% of the Chinese population within a comparatively short time of their introduction into that country (Lang 2001). The huge sums of money spent on direct advertising and promotions cost the food industry in the US about US$ 30 billion each year and all of this is to get people to consume more calories (Jacobsen *et al* 2000).

2.4.3. PHYSICAL ACTIVITY

A low level of activity generally contributes to ill health. Today's environment is designed to promote physical inactivity; for example, television, cars, elevators, internet shopping and delivery are increasingly prevalent. Western populations today are almost sedentary compared with their very active ancestors. In Southern Africa, although high levels of physical activity were previously characteristic in both African men and women, and in both

rural and urban areas, levels have now decreased, particularly in the latter areas probably due to an increased tendency to sedentary activities (Walker 1998).

Studies have shown that exercise facilitates weight reduction, improves insulin sensitivity, and decreases blood pressure. (Sowers *et al*., 2004) Exercise and insulin resistance exhibit an inverse relationship. Increased physical activity can cause a decrease in blood pressure and improve insulin sensitivity through several mechanisms like decreasing sympathetic nerve activity and improving vasodilatation as a result of an increase in the release of endothelium-derived nitric oxide (Whaley *et al* 1999).

2.4.4. SOCIOECONOMIC STATUS

Obesity has always been associated with the better circumstanced. It is now, however, becoming much more common among the poor (Kopleman 1999). In a review of some studies published before 1989 it was concluded that socio-economic status (SES) showed a consistent positive relationship to obesity in developing countries (Sobal *et al* 1989). Another review on studies conducted between 1989 and 2003 from developing countries portrayed a different picture. This review concluded that firstly, obesity in the developing world can no longer be considered solely a disease of groups with higher SES. Secondly, it showed that the burden of obesity in each developing country tends to shift towards the groups with lower SES as the country's gross national product increases. Finally, it showed that the shift of obesity towards women with low SES occurs at an earlier stage of economic development than it does for men (Monteiro *et al* 2004).

2.4.5. CULTURE

In African culture, obesity in women is regarded with far less disfavour than in the case of white women (Walker 1998). Accordingly, there is only limited incentive in obese African women to reduce their weight, except among urban dwellers that are better educated and in higher socio-economic circumstances. Also, in most of Africa, especially southern Africa, HIV/AIDS is readily associated with a lean body mass. Overweight or obese men and women in most communities therefore prefer to maintain their body masses and avoid stigmatisation associated with the disease.

2.5 COMPLICATIONS OF OBESITY

Some studies had indicated that increasing body weight is associated with higher mortality; a relationship that remained controversial until the results of a large prospective study involving over a million men and women in North America was published, strengthening the association of obesity with mortality (Calle *et al* 1999). The study revealed death rates from all causes (cardiovascular, cancer or other diseases) increased throughout the range of moderate and severe overweight for both men and women in all age groups. Although there are numerous complications associated with obesity, only those related to cardiovascular complications are discussed in this section. Any attempts to discuss all the complications associated with obesity are considered as beyond the scope of this work.

2.5.1. HYPERTENSION

The relationship between obesity and hypertension is widely recognized as studies have shown that weight gain increases blood pressure while weight loss has been shown to be effective at lowering blood pressure (Hall *et al* 2002; Hall *et al* 2001; Jones *et al* 1994). Results from the Framingham Heart Study for example, suggest that approximately 65% to

75% of the risk for hypertension can be directly attributed to excess weight (Jones *et al* 1994).

The two mechanisms (haemodynamic changes and impaired pressure natriuresis) have been proposed for how hypertension results from the obesity state. This has been summarised in figure 2.1

2.5.1.1 Haemodynamic changes

Obesity is associated with increased regional blood flow to the extra fat tissue and increased cardiac output (Hall *et al* 2002; Rocchini 1990). The blood pressure increase in obesity is related to the marked increase in cardiac output related to volume expansion from increased retention of sodium (discussed below) and from an increased sympathetic activity. Blood flow to non- adipose tissue (kidney, skeletal muscle etc) also increases with weight gain.

2.5.1.2 Impaired renal-pressure natriuresis

In obesity, hypertension is associated with a reduced capacity of the kidneys to lose sodium (impaired pressure natriuresis) (Rocchini 1990). This is initially due to an increased renal reabsorption of sodium because the glomerular filtration rate (GFR) and renal plasma flow are increased. Increased sodium reabsorption will thus result in volume expansion and elevated blood pressure (Hall 1997).

With prolonged obesity, increases in arterial pressure, renal vasodilation and glomerular hyperfiltration, neurohumoral activation, and metabolic changes may cause glomerular injury and further impairment of renal-pressure natriuresis, resulting in more severe hypertension and a gradual loss of kidney function (Hall 1997).

There are 3 mechanisms that have been clearly shown to mediate this increased reabsorption of sodium in the kidneys: (a) increased renal sympathetic activity, (b) activation of the renin-angiotensin aldosterone system and (c) altered intrarenal physical activities.

2.5.1.2.1 *Increased Renal Sympathetic Activity*

Several studies have shown that excess weight gain is associated with increased sympathetic activity, especially in the kidney (Landsberg *et al* 1989; Esler 2000). In experimental dogs for instance, it has been shown that increased sympathetic activity appears to raise blood pressure mainly through the renal sympathetic nerves (Kassab *et al* 1995).

Although the mechanism for the increased sympathetic activity in obesity is not clear, hyperleptinaemia has been proposed as one way through which obesity may increase sympathetic activity (Hall *et al* 2001). Both acute and chronic infusions of leptin have been demonstrated to cause sympathetic activation and to chronically sustain elevated blood pressure (Haynes *et al* 1997; Shek *et al* 1998). How increased leptin does this is also not clearly understood but it is suggested that important interactions with other neurochemicals in the hypothalamus may be the mechanism for leptin induced sympathetic overdrive. (Details on leptin, its biology, signalling pathways and relationship to CVDs, especially kidney diseases will be discussed in chapter 5).

2.5.1.2.2 *Activation of the Renin-Angiotensin-Aldosterone System (RAAS)*

Obese subjects have increased plasma renin activity (PRA), plasma angiotensinogen, angiotensin-converting enzyme (ACE) activity, and plasma angiotensin-2 (ANG II) levels despite marked sodium retention and an expanded extracellular fluid volume. A significant role for ANG II in stimulating sodium reabsorption, impairing renal-pressure natriuresis, and

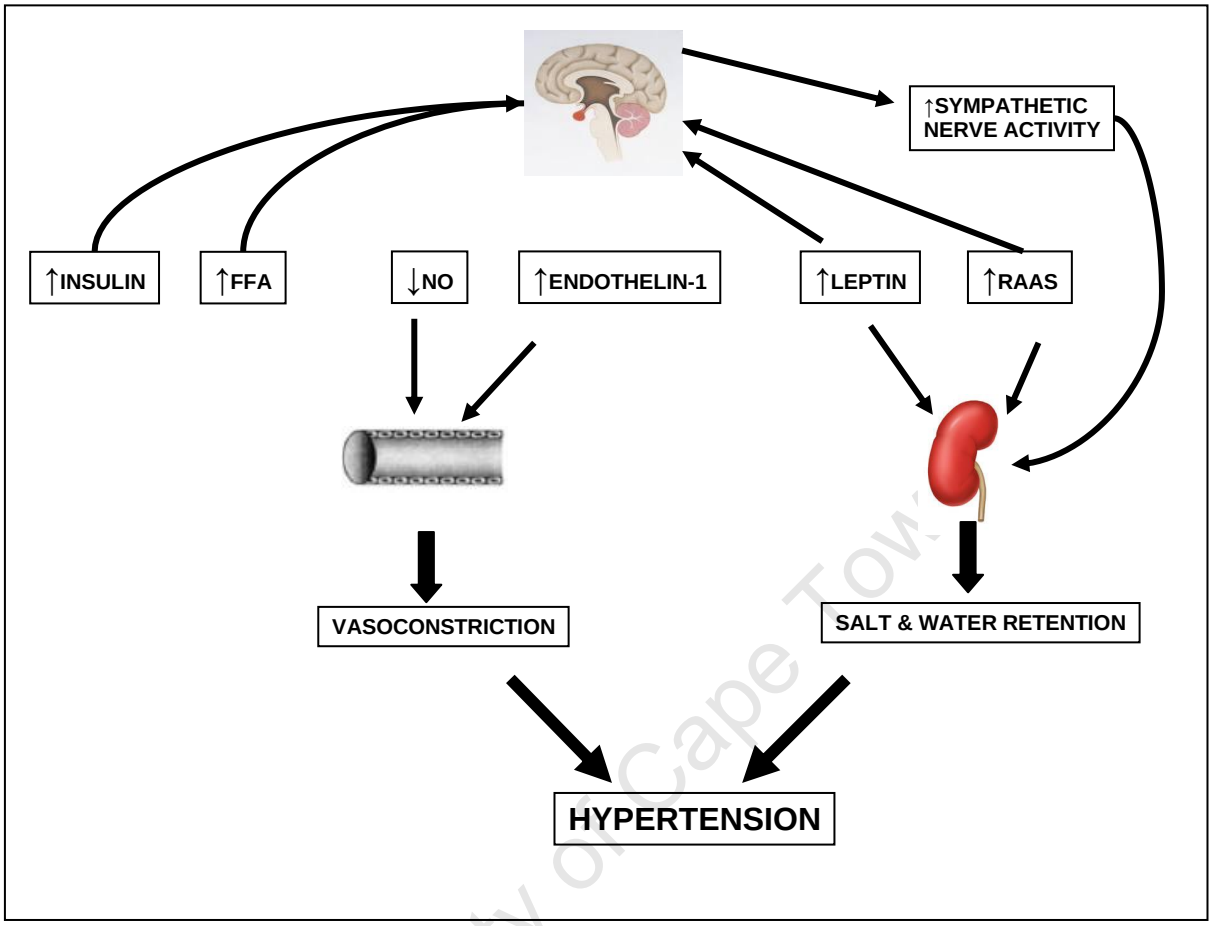
causing hypertension in obesity has been supported by the reports that treatment of obese dogs with an ANG II antagonist or ACE inhibitor blunts sodium retention and volume expansion, as well as increased arterial pressure (Hall *et al* 1997; Robles *et al* 1993).

ACE inhibitors are also effective in reducing blood pressure in obese humans, particularly in young patients (Reisen *et al* 1997). In addition to raising blood pressure, activation of the RAAS may also contribute to glomerular injury and nephron loss associated with obesity because increased ANG II formation constricts the efferent arterioles and exacerbates the rise in glomerular hydrostatic pressure caused by systemic arterial hypertension (Hall *et al* 1999).

2.5.1.2.3 ***Altered Intra-Renal Physical Forces.***

The intra-abdominal pressure of obese subjects is increased, reaching levels as high as 35 to 40 mmHg in some subjects with central obesity (Sugerman *et al* 1997). The kidney is retroperitoneally positioned and almost completely covered by adipose tissue that also penetrates into the medullary sinuses causing compression and increased intrarenal pressures (Hall *et al* 2002). It has been suggested that these increased intrarenal and intra-abdominal pressures may impair pressure natriuresis in the kidneys and contribute to obesity-associated hypertension (Hall *et al* 2002).

Figure 2.1: Summary of mechanisms and hormonal systems involved in obesity related hypertension (Adapted from Rahmouni *et al* 2005).



FFA; Free fatty acids, NO; Nitric oxide, RAAS, Renin angiotensin aldosterone system.

2.5.2. DIABETES

The link between obesity prevalence and rates of diabetes in different populations was reported by West (1978) who demonstrated that the prevalence of type 2 diabetes rises as the population becomes more obese. Data from NHANES has shown that for each kilogram increase in weight of the population the risk of diabetes increased by 4.5% (Ford *et al* 1997) and more recent data have even shown that in the US there is a 9% increase of risk of diabetes with each kilogram body weight gain (Mokdad *et al* 2000).

The mechanism of the occurrence of diabetes mellitus arising from obesity is summarised in figure 2.2. The adipose tissue, however, is increasingly being considered not only as an energy storage tissue but as an endocrine organ that communicates with the brain and peripheral tissues, secreting several hormones that regulate appetite and metabolism (Kershaw *et al* 2004). This endocrine function appears to be modulated by a number of factors relating to the adipose tissue.

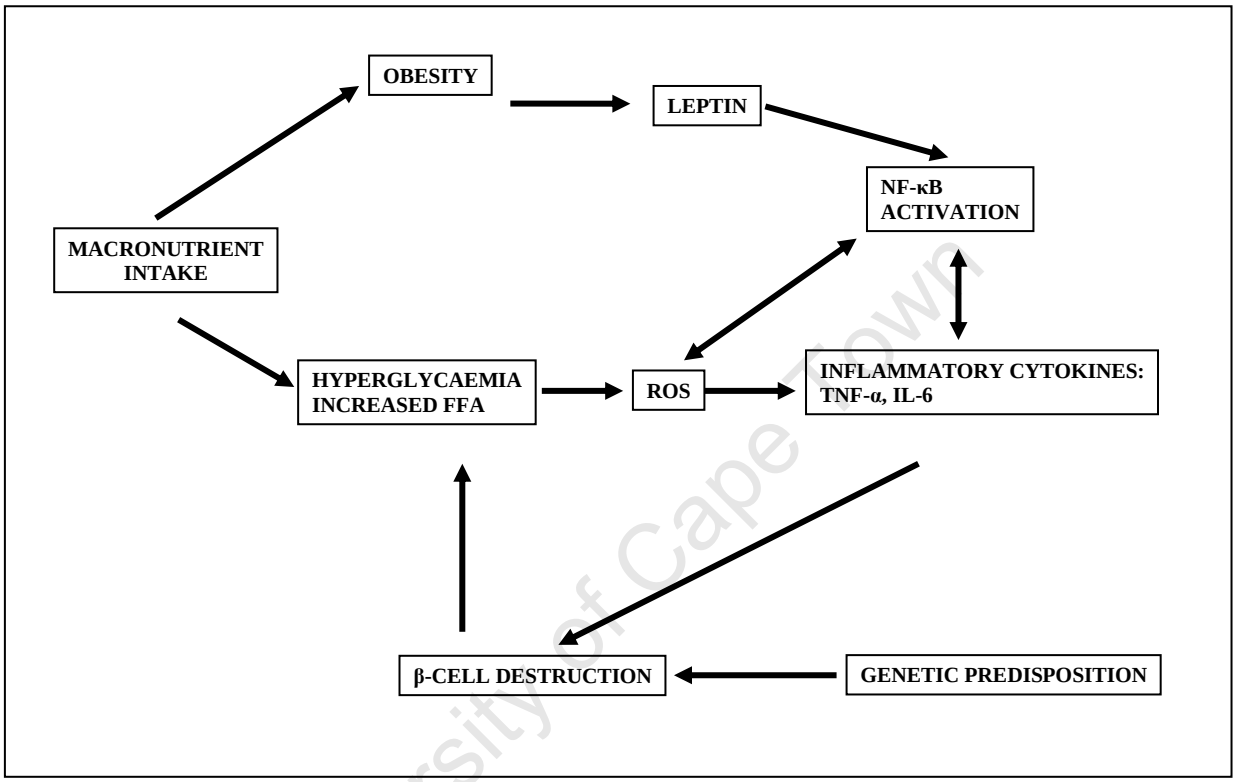
The first is the location of the adipose tissue; that is whether the adipose tissue is visceral or subcutaneous. It has been reported that of the approximately 1,660 genes that are expressed in fat tissue, 17.9% of these genes showed at least a twofold increase in their expression in visceral fat compared to subcutaneous fat. Many of these genes are involved in glucose homeostasis and insulin action or in lipid metabolism (Atzmon *et al* 2002).

Secondly, the relative size of the average adipocyte in the tissue may determine if it could have an endocrine function. Large subcutaneous abdominal adipocyte size has been associated with insulin resistance, and was shown to predict the occurrence type 2 diabetes in Pima Indians (Weyer *et al* 2001).

The third factor relates to the metabolism of glucose and corticosteroids in adipocytes. Downregulation of the glucose transporter, (GLUT 4) that mediates insulin-stimulated glucose uptake in adipocytes and muscle has been shown to cause insulin resistance in mice and therefore increases the risk of developing diabetes (Abel *et al* 2001). The activity of 11β hydroxysteroid dehydrogenase type 1 (11β HSD-1) plays a pivotal role in determining intracellular glucocorticoid concentrations and has been suggested to serve as a tissue-specific amplifier of glucocorticoid action (Seckl and Walker 2001). Transgenic mice

overexpressing the 11β HSD-1 exhibited pronounced insulin-resistant diabetes, hyperlipidemia, and hyperleptinaemia (Masuzaki *et al* 2001).

Figure 2.2: Mechanism of obesity related beta-cell destruction (Adapted from Dandona et al 2004)

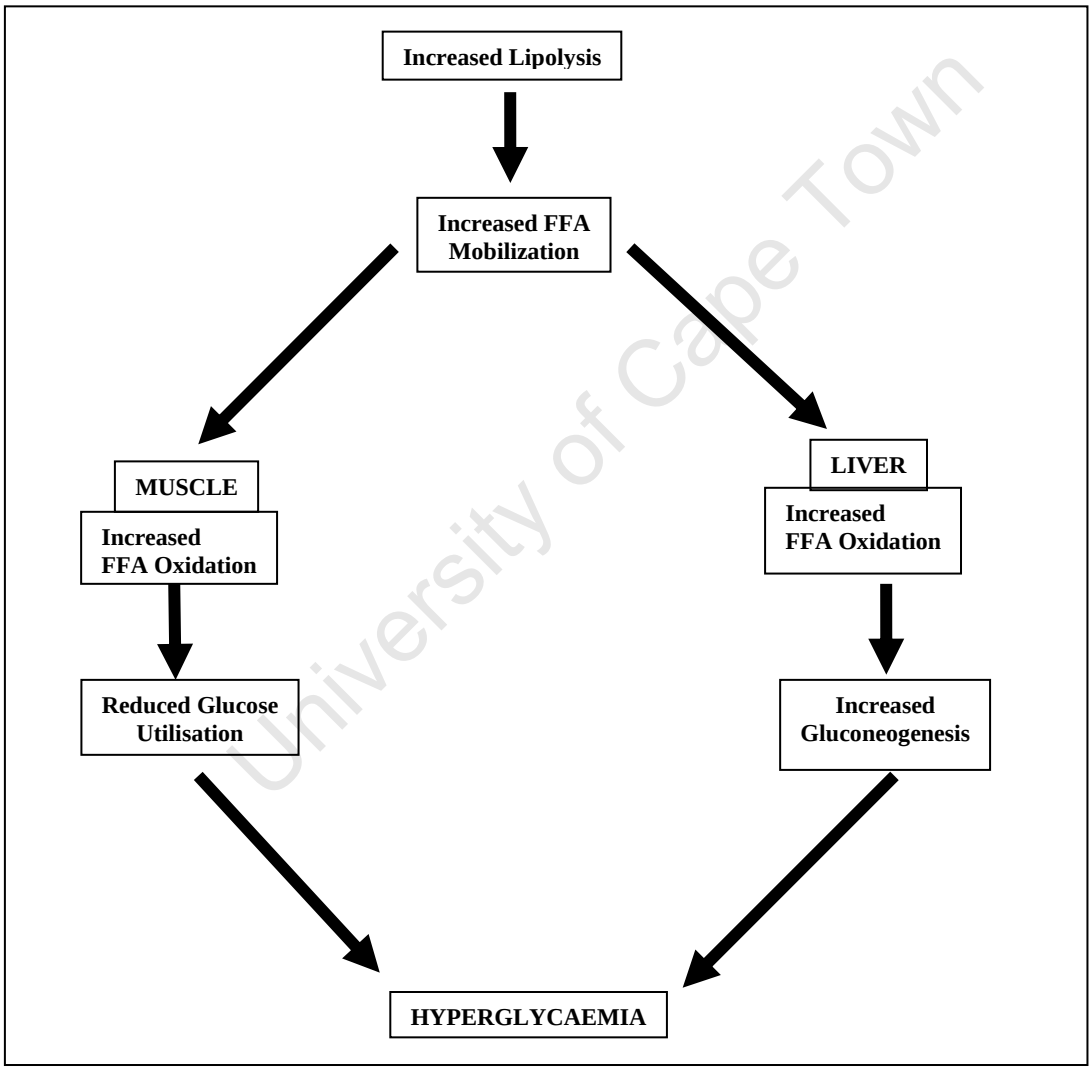


NF-κB - Nuclear factor kappa B, FFA – Free fatty acids, TNF – Tumour necrosis factor, IL – Interleukin.

Fourthly, the increased levels of free fatty acids (FFAs) found in obese individuals also contribute to the defects in glucose use and storage. Increased body fat leads to increased lipolysis with elevated FFA mobilization and as a result to increased FFA oxidation in muscle and liver. Glucose use begins to decline as FFA is used as the alternative fuel and hepatic glucose production increases in response to the higher FFA oxidation. These actions result in hyperglycaemia and impaired glucose tolerance (Figure 2.3).

Lastly, the adipose tissue also secretes a large number of proteins (cytokines) in addition to leptin that modulate glucose metabolism and insulin action (Kershaw *et al* 2004). Studies of mouse genetic models have established the causal role of some of these cytokines in insulin resistance and diabetes. Studies of humans generally suggest that circulating levels of these proteins are elevated in individuals with type 2 diabetes (Gabay *et al* 1999).

Figure 2.3: Free fatty acids induced hyperglycaemia via the skeletal muscles and the liver.



These proteins are known to play a role in innate immunity, for instance, tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) are produced by macrophages as well as by adipocytes and both act directly on inflammatory cells and also contribute indirectly to inflammation by acting on the liver to produce acute phase proteins (Gabay *et al* 1999). They induce suppressor of cytokine signaling-3 (SOCS-3), an intracellular signalling molecule that impairs the signalling of both leptin and insulin and SOCS-3 levels which are elevated in obesity and may represent a final common pathway of obesity-associated resistance to the actions of both leptin and insulin (Shi *et al* 2004).

2.5.3. INSULIN RESISTANCE AND HYPERINSULINEMIA

Reduction to insulin sensitivity can occur through an inherited defect, or as a consequence of obesity. The impact of obesity on insulin resistance independent of genetic factors is illustrated from a study of identical twins that were discordant for weight (Ronnemaa *et al* 1997). Within both male and female twin pairs, the obese member had higher fasting insulin levels and showed lower insulin sensitivity in the 75-g oral glucose tolerance test than the non-obese member and these differences were particularly evident among members with high abdominal fat distribution.

Once hyperinsulinemia and insulin resistance is acquired, it starts a cascade of metabolic changes that can lead to diabetes, dyslipidemia, hypertension, hypercoagulability, and eventually CVD. The relationship between insulin sensitivity and BMI is illustrated using the minimal model technique of Bergman (Bergman *et al* 1979). Khan and his colleagues (1993) applied this model to a group of non-diabetic subjects with widely varying BMI and found insulin sensitivity to be inversely correlated with BMI (Khan *et al* 1993).

In both the fasting and postprandial states, obese subjects require insulin levels that are several times higher than non-obese subjects to maintain normal glucose tolerance. At the cellular level, insulin normally binds to its receptor on the surface of target cells, thereby causing tyrosine auto-phosphorylation and consequent intracellular signalling. These events culminate in cellular responses, such as the translocation of glucose transporters to the cell surface to allow glucose uptake for use or glycogen storage. In obesity, however, insulin signalling is defective. Insulin stimulated protein kinase activity of the insulin receptor, which mediates tyrosine auto-phosphorylation, is reduced in obese subjects relative to non-obese ones, and it is further reduced in obese type 2 diabetes patients (Caro *et al* 1989).

2.5.4. CARDIOVASCULAR DISEASES

It has been stated that "no risk factor has as strong an impact on the cardiovascular risk profile as obesity" (Kannel 1997). Abdominal adiposity is particularly associated with CVD risk and obesity is a risk factor for unfavourable lipid profile: decreased high-density lipoprotein (HDL) cholesterol level, increased low-density lipoprotein (LDL) cholesterol and increased triglyceride levels all of which are related to CVD (Van den Hoogen *et al* 2000; Rexrode *et al* 1998).

American women from the Nurses' Health Study with BMI above 30 kg/m^2, had a threefold risk of developing nonfatal myocardial infarction compared with women with a BMI below 21 kg/m^2 (Manson *et al* 1990). Framingham data, based on 26 years of follow-up of approximately 5200 men and women aged 28–62, also showed that high relative weights were predictive of myocardial infarction, sudden death, congestive heart failure, and atherothrombotic strokes (Hubert *et al* 1983). It was estimated from the Framingham Study data that optimal weight, if achieved, would lead to 25% less coronary heart disease and 35%

fewer strokes or episodes of heart failure (Hubert *et al* 1983). Independent of other risk profiles like hypertension and lipid levels, obesity has also been shown to be directly related to CVD (Shaper *et al* 1997).

CHAPTER 3

THE METABOLIC SYNDROME

3.1 INTRODUCTION: HISTORY OF THE METABOLIC SYNDROME.

The first mention in literature on what is currently described as the metabolic syndrome (MS) was reported in the early 20th century by some European physicians who wrote about the relationship between metabolic disorders, blood pressure and diabetes (Hitzenberger and Richter- Quitter, 1921; Hitzenberger 1921; Kylin 1921; Maranon. 1922). Table 3.1 displays the names of authors and the year in which they reported on findings related to the MS.

Reaven, after several years of research on resistance to insulin-mediated glucose uptake, reported in 1988 that this disorder was present in the majority of subjects with type 2 diabetes mellitus or impaired glucose tolerance (IGT), but it was also present in 25% of the individuals with normal glucose tolerance. He thus formed the hypothesis that insulin resistance is the common aetiological factor for a group of disorders, consisting of IGT, hyperinsulinaemia, high levels of very low-density lipoprotein (VLDL)-triglycerides, low levels of high-density lipoprotein (HDL) cholesterol and hypertension. He then named this group of disorders "syndrome X" in an attempt to stress its unknown aspects and pointed out that individuals with the syndrome are at increased risk of atherosclerosis (Reaven 1988). Reaven's description of syndrome X was the spark that has since 'lit the fire' on the research of MS.

Table 3.1: Previous mentions of the metabolic syndrome in literature

Terms	Author and Year
Hypertension-Hyperglycaemia-Hyperuricaemia syndrome	Kylin, 1923
Metabolic trisyndrome	Camus, 1966
Pleurimetabolic syndrome	Avogaro and Crepaldi, 1967
Syndrome of affluence	Mehnert and Kuhlmann, 1968
Metabolic syndrome	Hanefeld and Leorhardt, 1981
Syndrome X	Reaven, 1988
Deadly quartet	Kaplan, 1989
Insulin resistance syndrome	DeFronzo and Ferrannini, 1991; Haffner, 1992

3.2 DEFINITION OF THE METABOLIC SYNDROME

There have been different definitions of the MS and the lack of a uniform definition from different expert groups essentially stems from different concepts about its origin and its importance in clinical practice. Opinions have varied as to whether it should be defined to indicate insulin resistance, defined for CVD risk stratification, or should simply reflect a collection of statistically related factors. Therefore, different expert groups have attempted to produce diagnostic criteria to define the MS being that its critical importance lies in identifying individuals at high risk of both type 2 diabetes and CVD.

The World Health Organisation (WHO) in 1998 first proposed a definition for the MS (Alberti and Zimmet 1998). The WHO criteria (Table 3.2) had insulin resistance or its surrogates (impaired glucose tolerance or diabetes) as essential components, together with at least two of: raised blood pressure, hypertriglyceridaemia and/or low HDL-cholesterol,

obesity (as measured by WHR or BMI), and microalbuminuria. The European Group for the Study of Insulin Resistance (EGIR) then produced another definition which was essentially a modification of the then existing WHO criteria by excluding people with diabetes mellitus (Balkau and Charles 1999). The EGIR criteria required hyperinsulinaemia to be present, used WC instead of BMI or WHR as measure of adiposity and had different cutoffs for other variables (Table 3.2).

In 2001, the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III) proposed a definition to facilitate clinical diagnosis and primarily aimed at defining CVD risk (NCEP 2001). The ATP III definition shifted from the previous two definitions by deemphasizing hyperinsulinaemia and insulin resistance, and required 3 of any 5 of: central obesity (measured as WC), raised blood pressure, raised triglycerides, low HDL-cholesterol, and fasting hyperglycaemia to be present in order to make a diagnosis of the MS (Table 3.2).

Due to these differences in definition, the confusion created from lack of specificity of the previous definitions, the inability to compare between studies carried out into the MS using these different definitions, and the view of the international diabetes federation (IDF) on the MS as the major driver of the worldwide epidemic of type 2 diabetes and CVDs, a new worldwide definition was then proposed in 2005 (IDF 2005). This new definition recommended the use of the diagnostic glucose level of the American Diabetic Association (ADA) for impaired fasting glucose (5.6mmol/L) to be used rather than the measurement of insulin resistance which was thought to be unrealistic. Waist circumference which had previously been linked to CVDs was the preferred measure of obesity with ethnic specific WC cut-offs proposed for different populations (Tan *et al* 2004). The ethnic specific WC

recommended reference to the individual's ethnic group, and not country of residence. The cutoffs for other variables of the MS were the same as those in the ATP III definition.

Finally, the IDF recommended some criteria to be used for research into the MS, including: measurement of insulin resistance, oral glucose-tolerance test, tests for endothelial dysfunction, imaging of visceral adiposity and liver fat, measurement of adipocytokines and inflammatory markers (adiponectin, leptin, IL-6, TNF-α), urinary albumin, and prothrombotic factors (plasminogen activator inhibitor type 1, fibrinogen) (IDF 2005). Since these recommendations were made, no groups have proposed any other definition for the MS rather the search now is for mechanisms causing it and leading to associated CVDs.

3.3 PREVALENCE OF THE METABOLIC SYNDROME

There are few national surveys reporting the prevalence of the MS, however available data suggest a wide variation from one population to another depending on the criteria used (Lakka *et al* 2002; Ford *et al* 2004; Gu *et al* 2005; Illanne-Parikka *et al* 2004). In the United States, data from the third NHANES of 1999-2000 using the ATP III criteria reported a prevalence of 27% (Ford *et al*., 2004) while the Kuopio study in Finland, carried out in non-diabetic middle aged men, free of CVD reported a prevalence of 8.8% by the ATP III criteria and 14.2% by the WHO criteria (Lakka *et al* 2002).

Reported prevalence tends to be dependent on the group studied within a given population (diabetics, specific gender, ethnicity/race, age groups). For instance, in a population based study of diabetic subjects in Italy, a prevalence of 75.6% was reported (Bruno *et al* 2004), while from the same country, when the study was carried out in subjects with diabetic complications; the prevalence had increased to 92.3% (Bonora *et al* 2004).

Table 3.2: Definitions of the metabolic syndrome as proposed by different expert groups.

Clinical index	WHO (1998)	EGIR (1999)	ATP III (2001)	IDF (2005)
Insulin resistance	IGT, IFG, T2DM, or Reduced Insulin sensitivity **plus 2 of :**	Plasma insulin > 75th percentile **plus any 2 of:**	None **Any 3 of following 5:**	None
Body weight	Men: WHR > 0.90; Women: WHR > 0.85 and/or BMI > 30kg/m2	Men: WC ≥ 94cm Women: WC ≥ 80cm	Men: WC ≥ 102cm Women: WC ≥ 88cm	Increased ethnic specific WC* **Plus any 2 of following:**
Lipids	TG ≥ 1.7mmol/L And / or	TG ≥ 1.7mmol/L And / or	TG ≥ 1.7mmo/L	TG ≥ 1.7 mmol/L or on treatment for elevated TG
	HDL: Men: < 0.9 mmol/L Women: < 1.0 mmol/L	HDL: Men: < 1.0 mmol/L Women: < 1.0 mmol/L	HDL: Men: 1.0mmol/L Women: 1.3 mmol/L	HDL: Men: 1.0 mmol/L Women: 1.3 mmol/L Or on HDL treatment
Blood pressure	≥140/90 mmHg	≥ 140/90 mmHg or on treatment for hypertension	≥ 130/85 mmHg	≥ 130mmHg systolic or ≥85 mmHg diastolic or on treatment for hypertension
Glucose	IGT, IFG, or T2DM	IGT, or IFG (Diabetes excluded)	> 6.1 mmol/L (includes diabetes)	≥ 5.6 mmol/L (diabetes included)
Others	Microalbuminuria	Nil	Nil	Nil

* See Table 2.2 for ethnic specific waist circumferences.

Gender differences in prevalence have been reported from several studies carried out in different populations (Illane-Parikka *et al* 2004; Oh *et al* 2004; Ford *et al* 2004). However, prevalence usually increases with age in men and women irrespective of race or ethnicity. The InterASIA study of 15,540 adults in China (Gu *et al* 2005) reported higher prevalence in women than in men in every age group, rising from 8.4% in men aged 35 to 44 years to 28.6% in women aged 65 to 74 years.

Significant variation in MS prevalence has also been reported among the different ethnic groups in the United States (Park *et al* 2003(a); Ford *et al* 2002). The age-adjusted prevalence of the MS from the NHANES III was 31.9% among Mexican Americans, 23.8% among non-Hispanic whites, and 21.6% among African Americans (Ford *et al* 2002). Data from the Third NHANES (NHANES III, 1988-1994) indicated that Mexican American men and women have the highest prevalence of the MS in the United States (Ford *et al* 2002). The age-adjusted prevalence of the MS was 31.9% among Mexican Americans, 23.8% among non-Hispanic whites, and 21.6% among African Americans (Ford *et al* 2002).

There have been far fewer studies reporting prevalence of the MS from Africa compared to studies from Europe, North America and South East Asia. However, available reports have also had differences in reported prevalence, depending on which definition is used and the specific population studied. Two studies from Nigeria using type 2 diabetics reported prevalence of 25.2% (Alebiosu and Odunsan 2004) and 59.1% (Isezuo and Ezunu 2005) even though the WHO criteria was used in defining the condition. The discrepant prevalence rates obtained from the two studies from Nigeria could be related to differences in ethnicity of the studied populations.

3.4 PATHOGENESIS OF THE METABOLIC SYNDROME

The aetiology of the MS is still not clear yet and likely to be complex in nature. Poor nutrition, physical inactivity, and subsequent increases in body weight have often been blamed as causes of the MS. What precisely causes the MS and how it comes about will remain important research questions until definitive answers are found. At present, mechanisms leading to the MS revolve around 2 major theories: insulin resistance and obesity.

3.4.1 METABOLIC SYNDROME: THE INSULIN RESISTANCE MECHANISM.

Metabolic syndrome and insulin resistance are often used interchangeably and there is a strong case for insulin resistance being the primary cause of MS. Although there is lack of clinical evidence to show that reduction in insulin resistance in people with the MS will prevent CVD, insulin resistance is still thought to be at the core of the MS.

Evidence for the central role of insulin resistance in the development of the MS is supported by the Bruneck Study in Italy which examined the prevalence of insulin resistance among 4800 subjects aged 40–79 using the homeostasis model assessment (HOMA) method. In this study, the degree of insulin resistance correlated with the number of metabolic abnormalities, and when several abnormalities were clustered together, insulin resistance was almost always present (Nesto 2003).

The findings from the Bruneck study, however, do not explain the link between insulin resistance and most components of the MS. Mechanisms that have been proposed to connect insulin resistance to the MS revolve around three major themes: (1) Tissue and cellular

effects of mild to moderate hyperglycaemia, (2) tissue and cellular effects of compensatory hyperinsulinemia and (3) unbalanced pathways of insulin action as described further below.

Mild to moderate, sub-diabetic, post-prandial hyperglycaemia, may lead to a variety of effects usually associated with diabetes such as accelerated atherosclerosis via advanced glycosylated end products or via enhanced collagen formation (discussed further in the next chapter in relation to diabetic kidney disease). However, evidence for such an association is very weak (UKPDS. 1998). Hence a more important mechanism for this effect may be the accompanying compensatory hyperinsulinaemia and the unbalanced pathways of insulin actions that results.

Insulin is normally secreted from the beta cells in response to post-prandial hyperglycaemia; the secreted insulin stimulates muscle uptake of the ingested glucose and suppresses gluconeogenesis in the liver. However, in insulin resistance, the hepatic and myocyte actions of insulin become compromised and the resultant hyperglycaemia turns into a continuous stimulus for insulin secretion.

Insulin binds to and activates its receptor through the phosphorylation of tyrosine phosphate residues thus initiating the intracellular signalling cascade (Figure 3.1). The two major pathways for insulin signalling are the phosphatidylinositol 3-kinase (PI-3K) and the mitogen-activated protein kinase (MAPK) pathways (Le Roith and Zick 2001).

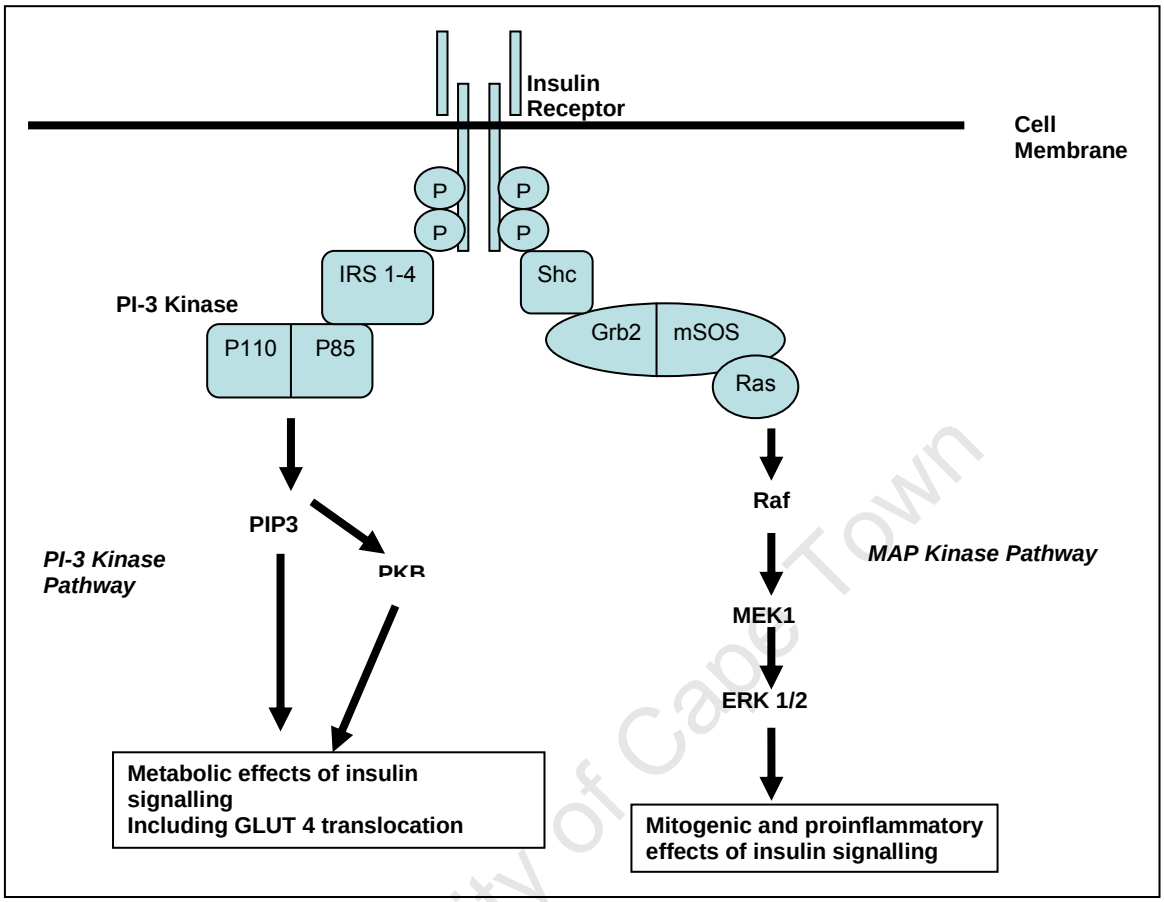
The PI-3K pathway is initiated by tyrosine phosphorylation of a member of the insulin receptor substrate family (IRS-1/2/3/4), which associates with the p85 regulatory subunit of PI-3K, leading to activation of the enzyme. Phosphatidylinositol 3-kinase causes

phosphatidylinositol 3, 4, 5-phosphate (PIP3) to be produced, resulting in the activation of protein kinase B (Akt) and other downstream effector molecules that mediate the metabolic response to insulin, which includes translocation of GLUT4 to the membrane (Cusi *et al* 2000).

The MAPK pathway begins with phosphorylation of Src homology 2 domain-containing (SHC) or insulin receptor substrate, which binds Grb2 and activates Ras via mammalian Son of Sevenless (mSOS). Ras then binds and disinhibits Raf, which activates another kinase, MEK1. MEK1 activates extracellular signal-regulated kinases ERK1 and ERK2. Activated ERKs, which are a type of MAPK, mediate the mitogenic and proinflammatory responses of insulin signalling (Cusi *et al* 2000).

In MS and type 2 diabetes mellitus, the pathways leading to activation of PI-3K are blocked, possibly through serine phosphorylation of the insulin receptor and/or IRS proteins, whereas the MAPK pathway remains open and may even be hypersensitive (Cusi *et al* 2000). Thus, antagonists of the appropriate serine kinase might reverse insulin resistance and restore the balance of MAPK and PI-3K signalling pathways. This has been demonstrated by Potenza *et al* who administered Rosiglitazone to spontaneous hypertensive rat (SHR) models of the MS. After three weeks of treatment, blood pressure, insulin levels, and ET-1 levels were lower, and adiponectin levels and insulin sensitivity were increased, with increased vasodilator response to insulin, consistent with rebalancing between PI 3-kinase and MAPK branches of insulin signalling (Potenza *et al* 2005).

Figure 3.1: Two major pathways of insulin signalling (Adapted from Miranda *et al* 2005).



Activation of the PI-3K signalling pathway also results in increases of cellular levels of nitric oxide, which is a potent inhibitor of vascular smooth muscle cell growth. Thus, an impairment in the PI-3K pathway could contribute to vascular endothelial dysfunction. Conversely, smooth muscle cell growth and proliferation are stimulated by activation of the ERK-MAPK pathway, which maintains normal sensitivity to insulin even in insulin-resistant conditions (Hsueh and Law 1999).

The overall effect of this unbalanced insulin pathway which creates a condition favourable to vascular endothelial dysfunction, smooth muscle cell growth and proliferation may well be to enhance atherogenesis and features of the MS (Cusi *et al* 2000; Hsueh and Law 1999).

3.4.2 THE METABOLIC SYNDROME: THE FAT (OBESITY) MECHANISM.

The purpose of adipose tissue throughout the body is energy storage. Calories are stored as triglycerides and released as fatty acids when energy is needed. It is safest for the body to store triglyceride in small peripheral adipocytes and if the capacity of these adipocytes to store triglyceride is exceeded, triglyceride accumulates in liver cells, skeletal muscle cells and visceral fat cells. This abnormal triglyceride deposition may lead to the development of hepatic and muscular resistance to insulin action. Excess triglyceride in myocytes and in abnormally large peripheral adipocytes appears to cause insulin resistance in these cells (Kelley and Mandarino 2000).

The lipotoxic effect of elevated FFA is thought to be the major mechanism through which obesity brings about features of the MS. However, this can occur through any or a combination of these processes: oxidative stress, proinflammatory signalling or through the actions of ceramide. Firstly, studies using magnetic resonance spectroscopy in humans have shown that increased fatty acid levels directly inhibit glucose transport by causing mitochondrial dysfunction (Lowell and Shulman 2005; Savage *et al* 2005) and stimulating certain protein kinases like PKC-theta, that then promote insulin resistance (Lowell and Shulman 2005).

Secondly, increased reactive oxygen species in response to fatty acids activates nuclear factor kappa B (NF-κB), which further stimulates the production of other proinflammatory cytokines, including TNF-α and IL-6 (Jove *et al* 2005; Boden *et al* 2005). TNF-α activates

signalling intermediates namely, IκB kinase beta (IKKβ) and Jun kinase 1 (JNK) which play a central role in cross-talk between inflammatory signalling and insulin signalling, leading to insulin resistance by phosphorylating IRS-1/2 on serine residues (Hirosumi *et al* 2002; Gao *et al* 2004). In other words, the process of inflammation triggered off by excess fat may be the cause of organ damage that results in the MS phenotypes.

Finally, ceramide, a product derived from long-chain saturated fatty acids inhibits insulin-stimulated activation of Akt and translocation of GLUT4 (Chavez *et al* 2003). Ceramide content in skeletal muscle of obese humans is increased, (Adams *et al* 2004) and overexpression of acid ceramidase protects against FFA-induced insulin resistance in vitro (Chavez *et al* 2005).

3.4.3 THE METABOLIC SYNDROME: OTHER MECHANISMS

The MS has been likened to Cushing syndrome which results from excess circulating glucocorticoids and manifests with hypertension, obesity, diabetes, and dyslipidaemia amongst other things. Peripheral fat is known to be shifted to central (visceral) depots by excess cortisol, hence the link between glucocorticoid excess and the MS. Activity and expression of 11- beta hydroxysteroid dehydrogenase type 1 (11-β HSD1), the enzyme responsible for the conversion of inactive cortisone to its active form cortisol, has been reported in adipose tissue of centrally obese women (Katz *et al* 1999; Engeli *et al* 2004). The expression levels of 11-β HSD1 were directly proportional to WC and insulin resistance (Engeli *et al*., 2004). Some studies have reported the relevance of locally formed glucocorticoids in white adipose tissue to the MS. Masuzaki and colleagues (Masuzaki *et al* 2001; Masuzaki *et al* 2004) described the occurrence of metabolic abnormalities and hypertension in transgenic mice overexpressing 11-β HSD1 in adipose tissues while Seckl

and his colleagues (2004) found that high-fat feeding failed to produce the MS in 11-β HSD1 deficient mice (Seckl *et al* 2004).

The hypothesis of foetal programming (thrifty phenotype) has come out of extensive epidemiological evidence, that is, that events occurring during foetal life permanently alter the structure and function of developing organs in the foetus with consequent predisposition to various metabolic diseases in adulthood (Barker 1994). Intrauterine growth retardation (IUGR) arising from foetal undernutrition can lead to permanently reduced nephron number (Brenner and Chertow 1994; Vehaskari *et al* 2001), permanently reduced pancreatic insulin-secreting β-cells (Holemans *et al* 2003), and permanently reduced skeletal muscle fibre number (Dwyer *et al* 1995) and mitochondrial mass (Park *et al* 2003b).

In the face of abundant nutrients, the "thrifty phenotype" (a smaller body size, a lowered metabolic rate and a reduced level of behavioural activity; adaptations to an environment that is chronically short of food) generates body size excess relative to organ capacity, deposits excess abdominal fat and promotes abdominal obesity, and the reduced pancreatic insulin secretory capacity may hasten transition from hyperinsulinemia to overt diabetes. Lower muscle mass—which persists in adults who experienced IUGR (Yajnik 2003)—could promote obesity via low basal metabolic rate and biologically based inactivity. The reduced skeletal muscle oxidative capacity and smaller mitochondria described in offspring with IUGR (Park *et al* 2003) could increase susceptibility to insulin resistance by favouring accumulation of myocyte FFA.

The "Birth-to-Ten Cohort" was a prospective study that investigated the relationship between birth weight and blood pressure and glucose tolerance in children from Soweto, South Africa

(Yach *et al* 1991). In this study, systolic blood pressure and blood glucose inversely correlated with birth weight at age 5years and 7 years respectively independent of current weight, maternal age, gestational age and socioeconomic status. Another study in Cape Town South Africa in a cohort of full-term offspring of primiparous women of mixed ancestry revealed significantly higher blood pressure and prevalence of impaired glucose tolerance in those with low birth weights compared to those with normal weights (Levitt *et al* 2000).

This chapter has reviewed the MS from the perspective of its different definitions and rising prevalence, and has attempted to explore its origins with a view to linking it to obesity. The clinical manifestations and complications of the MS are obviously beyond the scope of this work. The role of adipocyte hormones in inflammation will be discussed in chapter 5. The next chapter will discuss kidney disease as a model of CVD arising from obesity and the MS; reasons why hypertension alone cannot be blamed for kidney disease in black Africans will be the highlight.

CHAPTER 4

CHRONIC KIDNEY DISEASE

4.1 INTRODUCTION

Chronic kidney disease (CKD) is defined by the presence of sustained abnormalities of renal function and results from different causes of renal injury. CKD can lead to progressive loss of renal function, and may terminate in end-stage renal disease (ESRD) after a variable period of time following the initiating injury (NKF 2002). The public health impact of ESRD has led to increased interest in clinical and public health interventions that can delay or prevent the occurrence of ESRD in individual patients and in high-risk populations with CKD.

The National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF-KDOQI) Clinical Practice Guidelines for Chronic Kidney Disease recommend that CKD be adopted to define the presence of kidney injury and impaired kidney function (NKF 2002). The National Kidney Foundation criteria includes the presence for 3 or more months of impaired renal function across a continuum of renal injury from isolated anatomic, radiographic, biomarker, and urinary abnormalities to decreased glomerular filtration rate (GFR), irrespective of the primary cause of the renal injury (NKF 2002). Classification of CKD requires the establishment of presence or absence of renal injury, estimate of GFR, and determination that kidney disease has persisted for 3 or more months. Equations that convert the serum creatinine into an estimated GFR or creatinine clearance are available and are used to avoid misinterpretation of serum creatinine values.

An estimated GFR above 60 mL/min/1.73 m^2, in the absence of other anatomic, radiographic, or urinary abnormalities, is not classified as CKD. The NKF classification defines five stages of CKD by increasing degree of impaired kidney function (Table 4.1), and as kidney damage progresses the remaining nephrons compensate for the reduction in nephron mass by increasing the single nephron filtration rate with this hyperfiltration promoting further injury (Brenner and Mackenzie 1997). At each stage, therefore, patients can benefit from measures that delay or prevent the progressive loss of renal function such as modification of medications with renal clearance, avoidance of nephrotoxins, and reduction of cardiovascular risk factors (St Peter *et al* 2003). Patients with CKD need to be monitored for progression to kidney failure, and patients who advance to CKD stage 3 require increased monitoring and control of hypertension, anaemia, renal bone disease, and nutrition.

Recognition and early referral of patients who advance to stage 4 and 5 CKD is important if the transition to ESRD treatment is to be successful. It is thought that for patients in these early stages of CKD, early recognition and administration of appropriate treatment may delay the onset of ESRD. For example, delayed referral for ESRD treatment has been associated with less than optimal vascular access placement, failure to manage renal bone disease and nutrition, poor anaemia control, impaired quality of life, and increased risk of severe hypertension, uremic symptoms, pulmonary oedema, and emergent dialysis (Arora *et al* 1999; Lameire and Van Biesen 1999; Ifudu *et al* 1999).

Table 4.1: NKF-KDOQI Classification of Kidney disease by varying levels of GFR. (NKF 2002).

Stage	Description	GFR (ml/min/1.73m^2)
1	Kidney damage* with normal or increased GFR	≥90
2	Kidney damage* with normal or mild decrease in GFR	60 – 89
3	Moderate decrease in GFR	30 – 59
4	Severe decrease in GFR	15 – 29
5	Kidney failure / ESRD	< 15

CKD is defined as either kidney damage or GFR<60 ml/min/1.73m^2 for ≥3 months.
* Kidney damage is defined as pathologic abnormalities or markers of damage, including abnormalities in blood or urine tests or imaging studies.
GFR – Glomerular filtration rate.

4.2 EPIDEMIOLOGY OF CHRONIC KIDNEY DISEASE:

4.2.1. CKD AS A PUBLIC HEALTH PROBLEM

Chronic kidney disease is a worldwide public health problem. According to the World Health Report 2002 and Global Burden of Disease (GBD) project, diseases of the kidney and urinary tract contribute to the global burden of diseases, with approximately 850,000 deaths every year and 15,010,167 disability-adjusted life years (Zimmet *et al* 2001). Globally, they are the 12th and 17th cause of death and disability respectively.

Patients with ESRD consume a disproportionate share of health care resources. The total cost of the ESRD program in the US was $22.8 billion in 2001, an 11.5 percent increase from 2000 (USRDS 2003). The projected number of ESRD patients by the year 2010 has been estimated to be 661,330 and the total Medicare ESRD program cost in excess of $28 billion

USD (USRDS 2000). Although such data are not available in Africa, available estimates from Cape Town South Africa show that the annual cost of dialysis treatment per patient is $25, 000 and only about 100 patients are allowed into the National renal replacement programme at a given time due to cost (From Dr MD Pascoe, head of the Nephrology Unit University of Cape Town).

Despite the magnitude of the resources committed to the treatment of ESRD and the substantial improvements in the quality of dialysis therapy, these patients continue to experience significant mortality and morbidity, and a reduced quality of life. In 2000 alone, 72,342 ESRD patients died and the survival probabilities for dialysis patients at 1, 2, 5 and 10 years were 80, 67, 40, and 19 percent, respectively (USRDS 2003). Moreover, 50 percent of dialysis patients have three or more comorbid conditions, the mean number of hospital days per year is approximately 14 per patient, and self-reported quality of life is far lower in dialysis patients than in the general population (McClellan *et al* 1991; DeOreo 1997).

4.2.2. PREVALENCE OF CKD AND ESRD

An estimate of the global burden of ESRD was provided in a survey which included 122 countries. It reported that the approximate number of patients on renal replacement therapy (RRT) is over 1.4 million and that more than 80% of these patients live in Europe, North America, and Japan (Moeller *et al* 2002). Reported prevalence rates of patients on RRT are generally used to assess the prevalence of ESRD.

There is a wide variation in prevalence rate, expressed as number of patients per million population (ppmp), among countries and several factors may be responsible for these differences. Firstly, there is a strong relationship between prevalence rate and per capita

income, and governmental infrastructure, as these can influence both the availability and quality of dialysis and transplantation services. The prevalence rate of RRT is 644 ppmp in the 15 countries of the European Union (Berthoux *et al* 1999), compared with a prevalence rate of 166 patients per million population in Central and Eastern European countries (Rutkowski 2002).

Secondly, the reported prevalence rates in poorer countries like those of Africa and Asia being far less than those of developed economies is generally related to poor infrastructure. In West Africa for instance, the prevalence of ESRD ranges from 1.6 ppmp in Ghana to 20 ppmp in Mauritania (Fogazzi *et al* 2003) and in the countries of North Africa that have better established renal replacement programs, it ranges from 30 ppmp in Libya to 186.5 ppmp in Egypt (Naicker 2003). Data from the South African Dialysis and Transplant Registry (SADTR) of 1994 revealed that 3399 patients (99 ppmp) were alive and on treatment for ESRD (SADTR 1994).

Thirdly, the maintainance and updating of renal registries is not standardised. Renal registries offer an important source of information on several aspects of CKD and are very useful in gaining insight on the burden of kidney diseases and allowing comparisons of incidence and prevalence of ESRD, mortality and morbidity, patient demographics and treatment modalities among countries (Stengel *et al* 2003a). Also, renal registries often record data of patients who are at the last stage of kidney disease with very little known about the prevalence of earlier stages of CKD and it has been acknowledged that the majority of individuals at early stage of CKD have gone undiagnosed and under-treated. However, several countries do not have registries and the content of national registries is often uneven, for example, some registries are updated yearly, whereas other registries are updated less frequently.

Fourthly, there are striking racial and ethnic differences in the incidence and prevalence rates of CKD and ESRD. In 1999, the incidence rates for ESRD in the United States were 237 ppmp in Caucasians, 953 ppmp in African Americans, 386 ppmp in Asian Americans and native Hawaiians and other Pacific Islanders, and 652 ppmp in American Indians and Alaska Natives. Furthermore, 10 percent of all new ESRD patients were Hispanic (USRDS 2001). There is also significant variability in the causes of ESRD among the various racial and ethnic groups. As an example, whereas diabetic nephropathy is the most common cause of ESRD in all racial/ethnic groups, hypertensive nephropathy is the cause of ESRD in 33 percent of African Americans, compared to less than 25 percent in all other racial/ethnic groups. The age and gender adjusted ratio (African American to Caucasian) of hypertensive ESRD is 6 to 1. African Americans, and to a lesser extent other racial and ethnic minorities, also have a disproportionately higher incidence rate of ESRD due to diabetes and glomerulonephritis compared to Caucasians, and these minority groups in the US tend to reach ESRD at a younger age than Caucasians (mean age 57 and 58 years, compared to 63 years) (USRDS 2001).

4.2.3. POPULATIONS AT HIGH RISK OF CKD AND PROGRESSION TO ESRD

A number of populations are considered to be at high risk of developing ESRD and include these four groups: (1) patients with diabetes mellitus, (2) patients with hypertension, (3) patients with CVD, and (4) family members of incident ESRD patients. There are however, several other risk factors (including those that are components of the MS) associated with progression of CKD to ESRD and these are also discussed here.

4.2.3.1 Diabetes mellitus.

Diabetes mellitus is the most common cause of ESRD reported by the USRDS for the United States population and it accounted for nearly 45% of all new cases of ESRD starting renal replacement therapy between 1996 and 2000 (USRDS 2002). High blood glucose level is the common factor in the initiation and progression of renal injury among diabetic patients. Renal cells are stimulated by hyperglycaemia to produce humoral mediators, cytokines, and growth factors that are responsible for the structural alterations such as increased deposition of extracellular matrix (ECM) and the functional alterations such as increased permeability of glomerular basement membrane or shear stress (Schena and Gesualdo 2005).

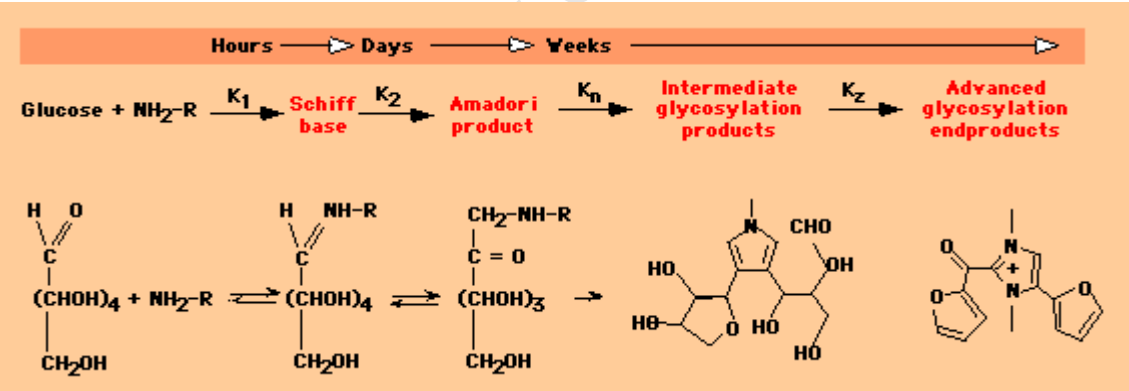
Hyperglycaemia additionally induces an abnormal activation of protein kinase C (PKC), which is involved in the development of diabetic nephropathy. Upregulation of PKC has been observed in the kidneys of rats with diabetic nephropathy (Koya *et al* 1997) and associated with transforming growth factor-β1 (TGF-β1), fibronectin, and collagen type IV upregulation.

Glucose Transporter-1 (GLUT-1), a surface receptor of resident renal cells, modulates the influx of glucose into renal cells. Mesangial cells have been shown to overexpress GLUT-1 mRNA when exposed to high glucose concentrations leading to overproduction of GLUT-1 protein and increased glucose transport into the cells (Heilig *et al* 1997).

Chronic hyperglycaemia can also result in glycosylation of serum and tissue proteins leading to the formation of advanced glycosylation end products. In this process, some of the excess

glucose combines with free amino acids on serum or tissue proteins, initially forming reversible early glycosylation products and later irreversible advanced glycosylation end products (AGEs) via an "Amadori" rearrangement (figure 4.1) (Vlassara 1996). Circulating and cellular AGE concentrations are increased in diabetic patients, particularly in those with renal insufficiency, since AGEs are normally excreted in the urine. This results in tissue accumulation of AGEs, in part by crosslinking with collagen, which can contribute to the renal and microvascular complications (Makita *et al* 1991). There is increasing evidence supporting a contribution of AGEs to the pathogenesis of diabetic kidney disease. Yang *et al* (1994) have shown that the administration of AGE-modified albumin to normal rats for four weeks led to glomerular hypertrophy and increased ECM production in association with activation of collagen, laminin, and transforming growth factor-β genes (Yang *et al* 1994).

Figure 4.1: Formation of advanced glycosylation end products in the presence of persistent hyperglycaemia (Rose and McCulloch. 2005).



4.2.3.2 Hypertension

Hypertension is ranked as the second most common cause of ESRD in the United States, accounting for 23% of incident ESRD patients between 1996 and 2000 (USRDS 2002). The higher USA incidence of hypertension-associated ESRD may relate to its higher incidence in African Americans. When compared with Caucasians, African Americans show a

disproportionate increase in the incidence of hypertensive ESRD in all age groups (USRDS 1999). In South Africa, hypertension accounted for 45.6% of all ESRD, second only to glomerulonephritis (52.1%) from the 1994 SADTR data (SADTR 1994).

Approximately 70% to 80% of individuals with CKD have hypertension, and its prevalence increases as glomerular filtration rate declines (Coresh *et al* 2001). Even mild-to-moderate hypertension is an important risk factor for progression of CKD toward irreversible renal failure (Coresh *et al* 2001). Typically, significant hypertension initially affects the renal vasculature, resulting in hyaline thickening of small arteries and arterioles. Eventually, the vascular lesions progress to vessel wall necrosis (fibrinoid necrosis, necrotizing arteriolitis and hyperplastic arteriolosclerosis), which may extend to the glomerulus as well (necrotizing glomerulitis) (Kumar *et al* 2003).

Mule and colleagues (2005) have analysed the influence of MS on target organ damage (cardiac, renal and retinal) in a group of non diabetic patients with essential hypertension. They found that the presence of the MS may amplify hypertension-related cardiac and renal changes, over and above the potential contribution of each single component of this syndrome (Mule *et al* 2005). Cuspidi *et al*, (2004) in a study of hypertensive subjects, reported equal values of ambulatory blood pressures in patients with and without the MS but found increased cardiac and extra-cardiac involvement including microalbuminuria in patients with the MS (Cuspidi *et al* 2004).

In the MS, hypertension is often related to obesity and is accompanied by impaired pressure natriuresis, renal vasodilation and glomerular hyperfiltration, neurohumoral activation, and metabolic changes all of which may cause glomerular injury and further impairment of renal-

pressure natriuresis, resulting in more severe hypertension and a gradual loss of kidney function (Hall *et al* 2002; Hall 1997).

4.2.3.3 Dyslipidemia

Lipid abnormalities are independent risk factors for the incidence and progression of CKD. Hunsicker *et al* (1997) described a systematic analysis to determine baseline factors that predict the decline in GFR, or which change the efficacy of the diet or blood pressure interventions in subjects that had participated in the Modification of Diet in Renal Disease (MDRD) Study. They found six factors including low serum HDL cholesterol to independently predict a faster decline in GFR (Hunsicker *et al* 1997).

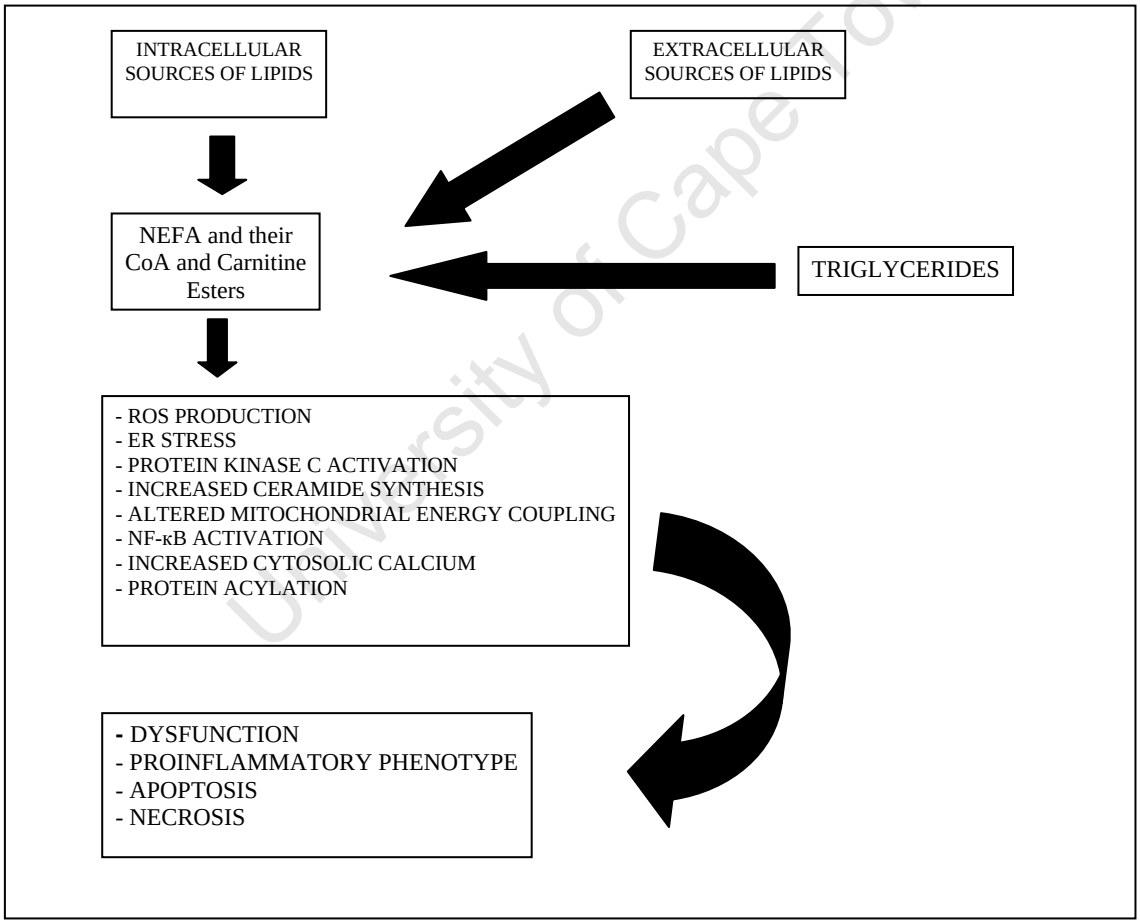
Lipotoxicity, mediated via long chain non-esterified fatty acids (NEFA), has been linked to many of the manifestations of the MS. High-circulating NEFA from abdominal fat mass drives the cellular uptake of more fatty acid, which inhibits the secretion of adiponectin and reduces the mitochondrial uptake and oxidation of FA. The excess intracellular FA is shunted toward the production of reactive intermediates, such as ceramide, diacylglycerol, and fatty acyl- CoA (Figure 4.2). These reactive compounds are cytotoxic and capable of inducing cell apoptosis and organ damage (Bagby 2004; Kamijo *et al* 2002).

In the kidney, filtered NEFA can aggravate the chronic tubule damage and inflammatory phenotype that develop during proteinuric states. Lipid loading of both glomerular and tubular cells is a common response to renal injury that contributes to the progression of nephropathy (Weinberg 2006).

4.2.3.4 Obesity

There is growing evidence that obesity may be a risk factor for progressive renal injury (Stengel *et al* 2003b; Fox *et al* 2004). The risk of either incident ESRD or kidney disease–related death among NHANES III participants was independently associated with a BMI greater than or equal to 35 kg/m^2 (Stengel *et al*., 2003b). Obese participants in the Framingham study who were initially free of kidney disease at baseline were more likely to have a decrease in estimated GFR (Fox *et al* 2004).

Figure 4.2: Cellular mechanisms of Lipotoxicity (Adapted from Weinberg 2006).



Obesity-associated renal dysfunction including proteinuria, nephrotic syndrome, and CKD are frequently seen in clinical practice (USRDS 2003). In a review of over 6800 renal

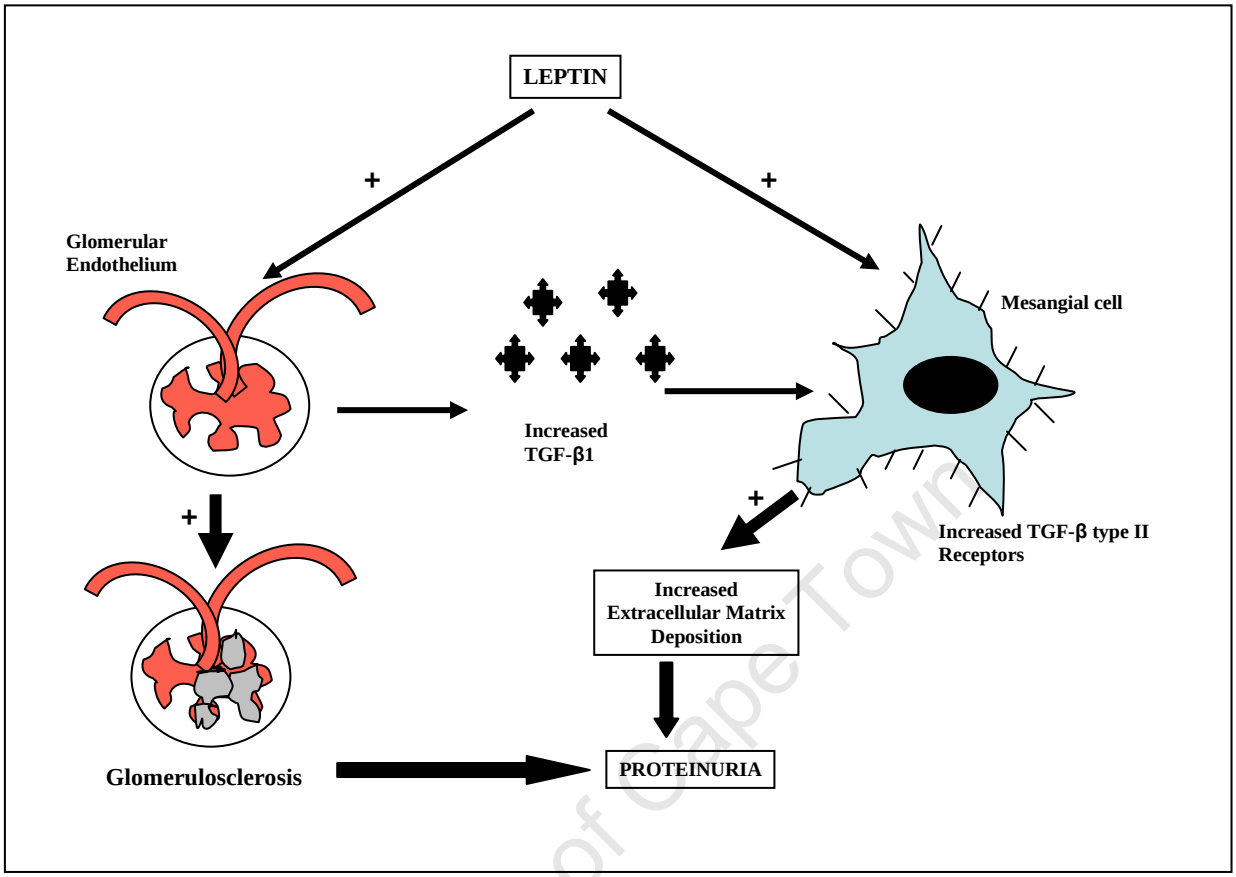
biopsies carried out in a study on obesity related glomerulopathy (ORG – defined as focal segmental glomerulosclerosis (FSGS) and glomerulomegaly or glomerulomegaly occurring alone), Kambham *et al* (2001) reported a progressive, 10-fold increase in biopsy frequency of ORG from 0.2% in 1986 to 1990 to 2.0% in 1996 to 2000 (Kambham *et al* 2001).

The first cases of obesity related FSGS in the paediatric population were reported in 2001 (Adelman *et al* 2001). Seven African- American adolescents with severe obesity were followed up over a 12 year period and had renal biopsies for the diagnosis of unexplained heavy proteinuria. Calculated creatinine clearance was normal in six patients and decreased in one. Observed histologic features included glomerular hypertrophy, FSGS, increased mesangial matrix and cellularity, relative preservation of foot process morphology, and absence of evidence of inflammatory or immune-mediated pathogenesis.

Compared with lean subjects, obese subjectss have reduced renal vascular resistance and increased renal blood flow, elevated GFR, and abnormal pressure natriuresis that shifts toward a higher blood pressure, often referred to as impaired pressure natriuresis (Reisin *et al* 1995; Reisin 2001). The increased activity of the sympathetic nervous system (SNS) and the renin-angiotensin-aldosterone system (RAAS), the blunted activity of the natriuretic peptides, accompanied with hyperleptinemia, and hyperinsulinemia, all contribute to renal sodium retention and a hyper-hemodynamic state in cases of obesity (Reisin 2001). Physical compression of the kidneys by adipose tissue is also thought to contribute to this complex interaction that ultimately gives rise to glomerular hyperfiltration, glomerular cell proliferation, matrix accumulation, and, finally, glomerulosclerosis and the loss of nephrons (Hall *et al* 1999).

Although the exact mechanisms that link obesity and renal damage have not yet been elucidated completely, it has been speculated that some of the many inflammatory cytokines that are secreted by adipose tissue may be involved in promoting renal impairment (Wisse 2004). Leptin is one of the many cytokines produced by fat cells and serum leptin levels and overall fat mass (Considine *et al* 1996) are positively correlated. Massively obese patients with hyperleptinaemia tend to develop FSGS (Kasiske and Crosson 1986). In the glomerular endothelial cells, leptin increases TGF-β1 synthesis and upregulates TGF-β type II receptor expression without influencing TGF-β1 synthesis in mesangial cells. TGF-β produced by endothelial cells may reach neighbouring mesangial cells and induce an amplified response because of upregulated TGF-β type II receptors (Figure 4.3). Leptin also stimulates the synthesis of type I collagen in mesangial cells and type IV collagen in glomerular endothelial cells. Activation of the TGF-β system by leptin eventually contributes to extracellular matrix deposition, glomerulosclerosis, and proteinuria (Ballermann 1999).

Figure 4.3: Transforming Growth Factor- beta (TGF-β) pathways between glomerular endothelial and mesangial cells mediated by leptin (Adapted from Wolf *et al* 2002).



4.2.3.5 Cardiovascular Disease

Patients with CVD and CKD are at increased risk of developing ESRD. Autopsy studies have found a correlation between extent of atherosclerosis and degree of glomerular scarring (Kasiske 1987; Smith *et al* 1989). In one such study, it was observed that the prevalence of glomerulosclerosis was substantially greater among individuals with moderate to severe atherosclerosis (15%) compared with individuals with mild disease (8%). Further, comparison of the mean glomerular area of nonsclerotic glomeruli suggested that there were compensatory increases in glomerular size in individuals with moderate to severe atherosclerotic disease and glomerular area was independently associated with increasing coronary artery atherosclerosis (Kasiske 1987).

4.2.3.6 Family members of patients with end-stage renal disease

ESRD aggregates within families (Ferguson *et al* 1988; Freedman *et al* 1997). An association between family history of ESRD and an increased risk of ESRD was first reported by Ferguson and his colleagues (Ferguson *et al* 1988) who noted that a family history of a first- or second-degree relative with CKD was reported by 26% of prevalent ESRD patients and 11% of community controls. A study in North Americans has reported that a family history of ESRD among first- or second-degree relatives is reported by 20% of incident ESRD patients in a single year (Freedman *et al* 1997). In that study there was substantial racial disparity in the frequency of a positive family history of ESRD among race-sex groups, with 14.1% of white men, 14.6% of white women, 22.9% of black men, and 23.9% of black women reporting a first- or second-degree relative with ESRD. A positive family history was reported more frequently among individuals with ESRD attributed to diabetes mellitus (22.2%) and hypertension (18.9%) compared with those without either diagnosis (13%). Patients reporting a positive family history were more likely to be black, to be younger, and to be better educated.

4.2.3.7 Urine protein excretion

The presence and degree of proteinuria are strong risk factors for progressive kidney disease in both diabetic and non-diabetic kidney disease (Peterson *et al* 1995; ACE Inhibitors in Diabetic Nephropathy Trialist Group 2001). A meta-analysis of patient-level data from 11 randomized, controlled trials found that the risk of doubling of baseline serum creatinine level or onset of kidney failure was independently associated with both blood pressure reduction and urine protein excretion less than 2 g/d and was associated with the lowest risk for kidney disease progression. Further, a higher level of urine protein excretion during

follow-up, determined by the difference between baseline and final levels of proteinuria, was found to increase the risk of progressive kidney disease by nearly sixfold for each 1 g/d higher protein excretion (ACE Inhibitors in Diabetic Nephropathy Trialist Group 2001).

4.2.3.8 Dietary protein consumption

It is now well established that restricting the amount of protein consumed in the diet delays the progression of CKD to ESRD. Wrone *et al* (2003) examined the association between dietary protein intake as assessed from 24-hour dietary recall and the prevalence of albuminuria among NHANES III participants and they reported that persons with both hypertension and diabetes and in the highest quintile of dietary protein consumption had over threefold increased risk for albuminuria compared with those in the lowest quintile of protein intake.

4.2.3.9 Smoking

Smoking is a well recognized risk factor for CVD, malignancy, and pulmonary disease. It is also a pleiotropic risk factor for genitourinary disease, including cancer, proteinuria, and CKD (Chuahirun *et al* 2004). A number of observational studies have reported that smoking cessation is associated with reduced risk of progressive renal injury, suggesting that smoking should be considered a risk factor for progressive renal insufficiency and cessation may be renoprotective and beneficial to general and cardiovascular health (Chuahirun *et al* 2004).

4.2.3.10 Race and Poverty

Blacks have a disproportionate risk of ESRD. In contrast to ESRD, an unequivocally disproportionate risk of CKD among blacks compared with whites has not been demonstrated (Hsu *et al* 2003). The reasons for the discrepant patterns of risk for prevalent CKD and

incident ESRD are not clear. It has been suggested that higher ESRD incidence rates among blacks could reflect a faster progression of CKD, and racial differences in the progression of CKD to ESRD have been reported in a study using NHANES III data (Hsu *et al* 2003). Among black and white NHANES III participants with a GFR between 15 and 59 mL/min/1.73 m^2 it was estimated that, despite comparable prevalence of CKD, the subsequent risk of ESRD for blacks during follow-up was fivefold.

There is growing evidence that racial disparities in the risk of progressive CKD may reflect socioeconomic, environmental, and behavioural factors. Black participants in the NHANES II survey had an 8.9-fold increase in risk of treated ESRD (Tarver-Carr *et al* 2002). After adjusting for age and gender 43.8% of the excess risk among blacks was explained by lifestyle and clinical risk factors that were potentially modifiable.

Whittle *et al* (1991) have reported the role of poverty and access to health care in racial differences in ESRD risk. An eightfold disparity in risk of ESRD among blacks compared with whites was noted before accounting for such factors like community specific characteristics where the individual resided before initiation of renal replacement therapy. However, after controlling for these factors and measures of socioeconomic and health care status there was a 5.5-fold reduction in risk.

There may also be racial differences in the response to protective benefits of antihypertensive therapy. Walker *et al* (1992) evaluated the rate of loss of renal function among white and black participants in the Multiple Risk Factor Intervention Trial (MRFIT). During follow up the mean rate of change in renal function as measured by serum creatinine for black subjects was -0.0090 ± 0.0013 mg/dl/yr compared with 0.0018 ± 0.0004 mg/dl/yr for whites. Blood

pressure control was similar for both blacks and whites. Factors associated with increased rate of change in serum creatinine included age and degree of blood pressure control. When the MRFIT participants were stratified by race, better blood pressure control was associated with slower loss of renal function in whites, but not in blacks.

4.3 WHY IS THERE A PREPONDERANCE OF HYPERTENSION RELATED ESRD IN BLACK AFRICANS?

Data from the USRDS has shown that hypertension is the most common cause of ESRD in America. Some have questioned the labelling of "ESRD secondary to hypertension" in dialysis registries as this is very often racially biased (Perneger *et al* 1995) and unsupported by valid histological proof (Luft 2000) or documentation to show that hypertension was present before the existence of evidence of renal disease (Schlessinger *et al* 1994).

In a study of 43 subjects referred for kidney transplant with a diagnosis of ESRD secondary to hypertension it was found that few of the patients had undergone kidney biopsy and that none of those who had undergone biopsy had the classic features of benign nephrosclerosis. Fewer than 5% of patients had hypertension documented at any time with normal renal function (Schlessinger *et al* 1994). In other words, it was not possible to determine whether renal disease or hypertension presented first. Interestingly, 86% of the patients in this study were black (versus 50% in the overall transplant referral population at that centre).

The possibility of over-diagnosis of "hypertensive nephrosclerosis" among African-Americans was explored in another study (Perneger *et al* 1995). The researchers sent written case histories of ESRD patients to practicing nephrologists and asked for "the primary underlying cause" of the renal disease. For each case history, the patient's race was randomly

assigned as “black” or “white”. The authors found that “black” patients were almost twice as likely to be given a diagnosis of hypertensive ESRD as “white” patients with identical case histories (Perneger *et al* 1995).

In a review, Seedat (1999) had noted that over a 10 year period, the incidence of stroke and myocardial infarction, 2 major complications arising from hypertension had reduced by about 25% in black Africans following effective treatment of hypertension but that this trend was not seen in ESRD. Factors suggested explaining this racial disparity of excess hypertension related ESRD included: misdiagnosis, socioeconomic status, higher prevalence and greater severity of diabetes and hypertension in black Africans and an increased inherited susceptibility of black Africans to kidney diseases (Seedat 1999; Krop *et al* 1999; Brancati *et al* 1992; Freedman *et al* 1991).

Although it is not fully known why people of African decent suffer more hypertension related kidney disease, an interesting mechanism to explain this racial difference in severity of hypertensive nephrosclerosis may be due to increased production of fibrogenic growth factors like transforming growth factor and platelet-derived growth factor in blacks (Dunstan 1995).

CHAPTER 5

THE ADIPOSE TISSUE AS AN ENDOCRINE ORGAN

5.1 INTRODUCTION:

The adipose tissue, previously only considered an organ for storage of energy is increasingly being seen as an endocrine tissue. There are two forms of adipose tissue: white adipose tissue (WAT) which stores energy in the form of triglycerides and secretes a vast amount of hormones or so-called adipocytokines that act in an autocrine, paracrine, or endocrine fashion to control various metabolic functions (Table 5.1) and brown adipose tissue (BAT) which serves primarily to dissipate energy.

Although it was initially suggested that insulin resistance and hyperinsulinemia are primarily responsible for the development of all components of the MS and for the atherosclerotic or pro-atherogenic effects of obesity (Reaven 1988), studies have shown that adipose tissue hormones are also involved in this process (Hotamisligil *et al* 1993).

Epidemiological studies have demonstrated that inflammation is an important link between CKD and CVD. Evidence suggests a fundamental role for innate and adaptive immunity in all stages of atherosclerosis and the pathogenesis of CVD as well as progression of CKD (Ross 1999). Patients with CKD have higher circulating levels of cytokines and abnormal cytokine metabolism, leading to elevated levels of acute phase proteins.

In CKD, once a certain number of the nephron mass is lost, 'nonspecific' glomerular and tubulointerstitial scarring begins and progresses, and this is seen in all forms of CKD. The final common pathway of mechanisms of this progression includes intraglomerular

hypertension, hyperfiltration in the remnant glomeruli, systemic hypertension, poor glycemic control (in diabetics), mesangial, endothelial, and epithelial injury from protein and macromolecular trafficking, and hyperphosphatemia. Inflammatory and profibrotic cytokines have also been shown to facilitate the progression of glomerular injury leading to CKD as they may modulate glomerular responses to infectious, dietary and environmental antigens (Ketteler *et al* 1995). In the kidney, cytokines induce (a) resident cells to proliferate, (Nakamura *et al* 1992) (b) aberrant matrix metabolism, (Coleman *et al* 1992) (c) procoagulant activity of endothelium, (Bevilacqua *et al* 1986) (d) reactive oxygen/nitrogen species, (Sharma *et al* 2005) and expression of (e) adhesion receptors, (Park *et al* 1998) bioactive lipids, (Zager and Johnson 2001) and metalloproteinase (Atkins 1995). These molecules may be the effectors through which the renin–angiotensin system and hemodynamic factors mediate their actions (Ketteler *et al* 1995).

Although the relationships between several of these vasoactive substances, growth factors and cytokines with CVD or CKD have been previously studied and reported, this section will focus on leptin being the hormone of interest to this thesis and having the ability to cause CVDs through the inflammatory process. The biology, receptors, pathways and evidence for its role in CVDs and CKD will be presented.

Table 5.1 Effects of proteins secreted by the adipocytes.

MOLECULE	EFFECT
Adiponectin	Improves insulin sensitivity, improves fatty acid transport and utilisation
Adipsin	Required for the synthesis of ASP, possible link between activation of complement pathway and adipose tissue metabolism
Agouti	Might be involved in inducing insulin resistance through increasing intracellular free calcium concentrations
Angiotensinogen	Regulator of blood pressure and electrolyte homeostasis
aP $_2$	Involved in intracellular trafficking and targeting of fatty acids
ASP	Activates diacylglycerol acyltransferase, inhibits hormone sensitive lipase, stimulates GLUT-4 translocation to the cell surface
IGF-1	Stimulates cell proliferation and mediates many of the effects of growth hormone
IL-6	Increases hepatic glucose production and triglyceride synthesis.
Leptin	Feedback effect on hypothalamic energy regulation; maturation of reproductive function
MIF	Involved in proinflammatory processes and immunoregulation
PAI-1	Potent inhibitor of the fibrinolytic system
PGI_2 and PGF_2 α	Implicated in inflammation and blood clotting, ovulation and menstruation.
Resistin	Appears to impair insulin sensitivity
TGF-ß	Regulates growth and differentiation of numerous cell types
TNF-α	Mediator of the acute phase response, inhibits lipogenesis, stimulates lipolysis and impairs insulin-induced glucose uptake, thus leading to insulin rsistance and weight loss

aP2 – Adipocyte protein 2, ASP – Acylation stimulating protein, IGF-1 – Insulin-like growth factor 1, IL-6 – Interleukin -6, MIF – Macrophage migration inhibition factor, PAI-1 Plasminogen activator inhibitor-1, PGI – Prostacyclin, $PGF_2\alpha$ – Prostaglandin F-2 alpha, TGF-β – Transforming growth factor beta, TNF-α – Tumour necrosis factor alpha.

5.2 THE BIOLOGY OF LEPTIN

Leptin was identified in 1994 by Zhang *et al* (1994) through positional cloning of the *ob* gene which determines the development of obesity in *ob/ob* mice. Leptin is the product of *ob* gene coding for a 167 amino acid protein with a 21 amino acid signal peptide (Zhang *et al* 1994). Its crystal structure indicates that leptin is a member of the cytokine family and has four helical segments (Madej *et al* 1995). In adult animals, leptin mRNA is primarily detected in WAT and BAT (Masuzaki *et al* 1996), however, it is also synthesized and secreted in a number of extra adipose tissues that include the gastric mucosa (Bado *et al* 1998), mammary epithelial cells (Smith- Kirwin *et al* 1998), myocytes (Wang *et al* 1998), the placenta (Senaris *et al* 1997), and the testes, ovary and hair follicles (Hoggard *et al* 1997).

Leptin circulates in the plasma as a free form or bound to leptin-binding proteins. The great majority of leptin circulates in the bound form in lean individuals and in the free form in obese subjects (Sinha *et al* 1996).

Leptin levels rapidly decrease during fasting and remain low until four to six hours after eating when they begin to rise again (Schoeller *et al* 1997). Plasma leptin levels show a diurnal pattern with a nocturnal peak shortly after midnight and a midmorning trough between 10 am and 12 noon (Sinha *et al* 1996b). Insulin also plays a role in the regulation of leptin secretion: prolonged insulin infusions markedly increase serum leptin levels (Boden *et al* 1997).

Leptin slowly declines during aging and this reduction is higher in women than in men (Isidori *et al* 2000). Leptin provides the brain with the information about the fat stores of the body and thus acts as a part of the feedback mechanism that can function as a lipostat. It has been suggested that when fat cells increase in number and size, the *ob* gene starts to produce

leptin, which is secreted into the circulation (Klein *et al* 1996). When leptin reaches the brain it decreases appetite and enhances the metabolic rate. In human subjects, a high correlation between the body fat content and plasma leptin concentrations has been found and data indicate that loss of body fat decreases leptin levels, which induces a state of positive energy balance and other adaptive changes (Ahima *et al* 1996).

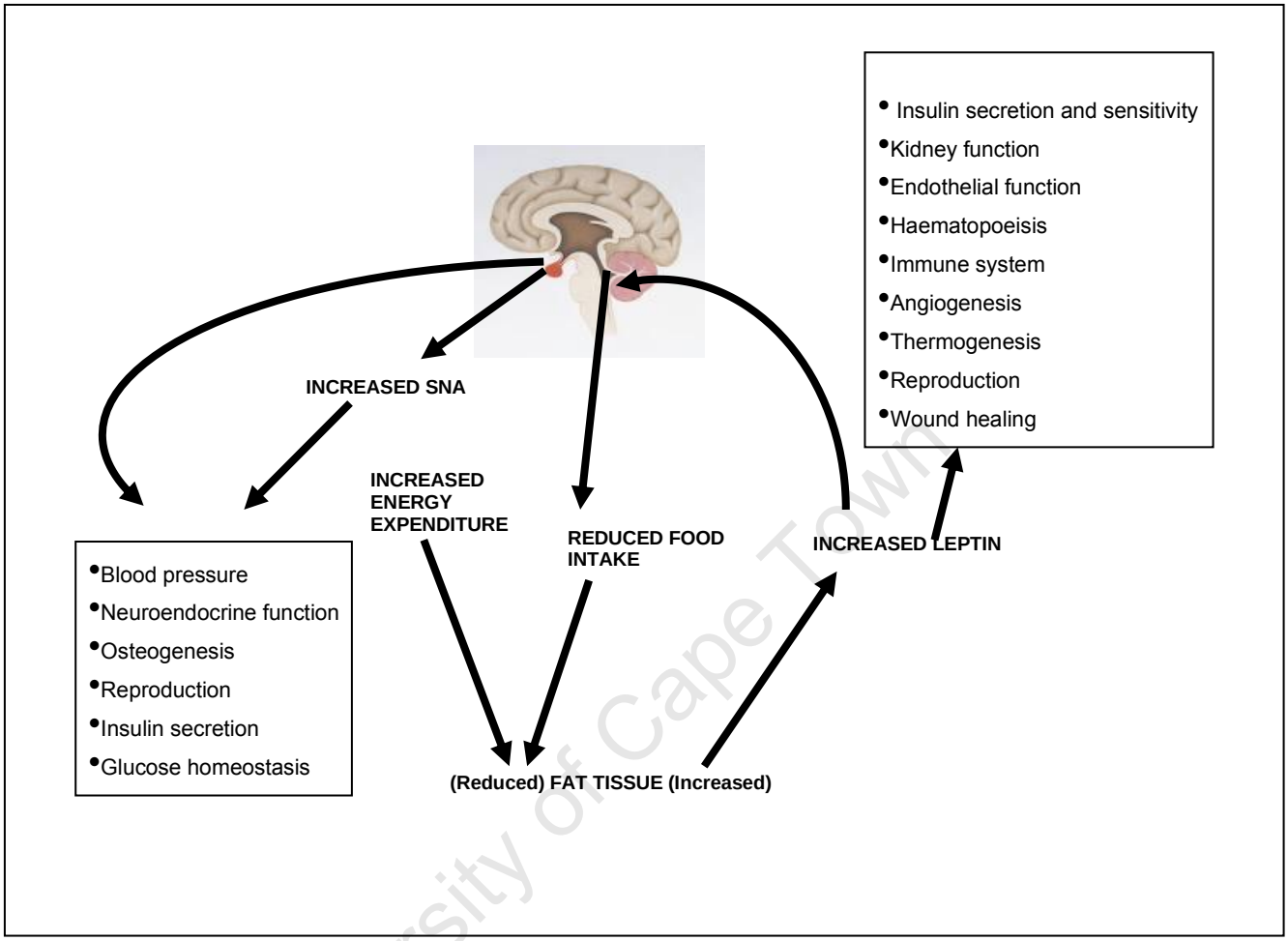
Although the main role of leptin is as an adipostat, recent studies have shown that leptin has a broad range of effects in different tissues (Margetic *et al* 2002). Leptin affects these different functions either by direct action in peripheral tissues or through its action in the CNS (Figure 5.1).

5.3 LEPTIN RECEPTOR AND MECHANISMS OF SIGNALLING

5.3.1 LEPTIN RECEPTORS

Leptin receptors are the product of the diabetes (db) gene, which is alternatively spliced to produce at least six isoforms; obRa–obRf (Figure 5.2). Each of obRa–obRd and obRf are identical in their extracellular and transmembrane domain. The obRe has no transmembrane or intracellular component and exits as a soluble receptor isoform, does not play a direct role in leptin signalling but is likely to be important in determining the amount of leptin in the circulation (Huang *et al* 2001). The other isoforms possess a transmembrane domain, which is comprised of 23 amino acids and the extracellular domain of the leptin receptor consists of 816 amino acids (White and Tartaglia 1996).

Figure 5.1: Role of Leptin in the regulation of body weight and other functions (Adapted from Rahmouni and Haynes 2004)



The obRb has a long intracellular domain whereas obRa, obRc, obRd and obRf all possess short intracellular domains (Figure 5.2). The intracellular domain of obRb is approximately 306 amino acids and in the short forms is 32–40 amino acids (Tartaglia *et al* 1995). The intracellular domain of all these isoforms contains an identical 29 amino acid sequence containing a 'Box 1' Janus-family tyrosine kinase (JAK) binding domain in the juxtamembrane region while obRb also contains a 'Box 2' motif and signal transducers and activators of transcription (STAT) binding sites. Human obRb contains five tyrosines, and each may be associated with the activation of distinct downstream signalling pathways (Banks *et al* 2000). Although obRb is traditionally viewed as the 'signalling isoform', there is

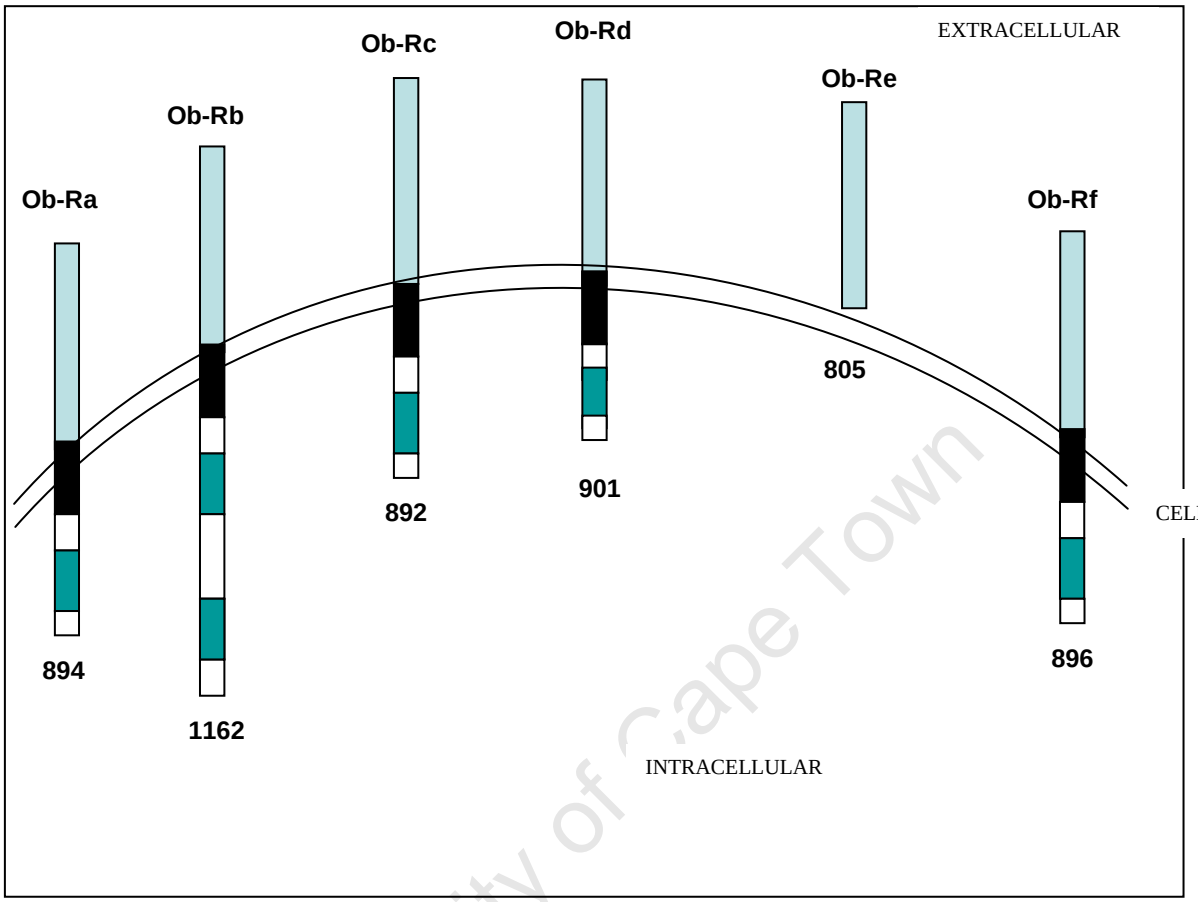
clear evidence that short receptor isoforms are capable of signalling and indeed show divergent signalling capacities (Bjorbaek *et al* 1998 b).

Expression of obRb is highest in hypothalamus (Fei *et al* 1997), yet, it is also found in many peripheral tissues at lower levels (Morton *et al* 1998). The obRa is expressed fairly ubiquitously and represents the major isoform of many peripheral tissues (Fei *et al* 1997). Other short receptor isoforms tend to be expressed at low levels. However, examination of obR isoform expression in various tissues by RT–PCR has often been inconsistent.

5.3.2 LEPTIN SIGNALLING PATHWAYS

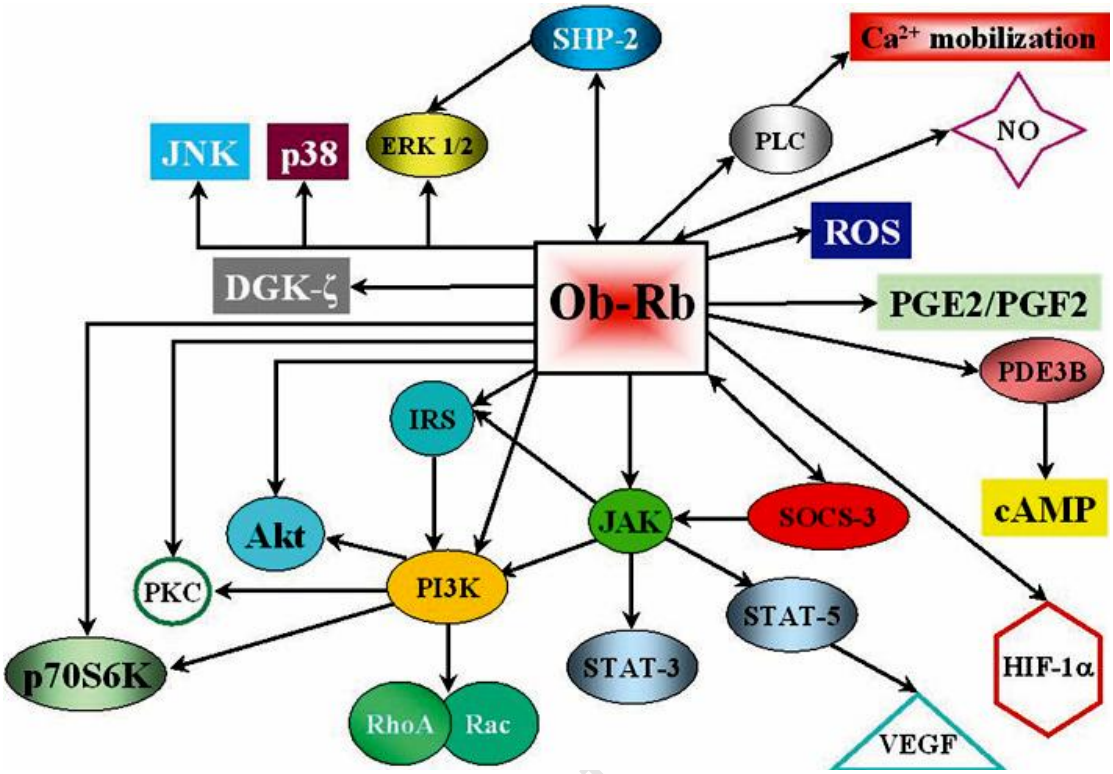
The signalling pathways regulated by leptin include: The Janus kinase / signal transducer and activator of transcription (JAK/STAT) pathway, Src-like homology 2 (SH2) domain containing protein tyrosine phosphatase (SHP-2) pathway, mitogen-activated protein kinases (MAPK) pathway, suppressors of cytokine signalling (SOCS) pathway, Phosphatidylinositol (PI) 3-kinase and insulin receptor substrate (IRS) proteins pathway. Others are the protein kinases (PKB, also called Akt and PKC), cyclic AMP PDE, Nitric oxide (NO) and the Rho family proteins (Figure 5.3).

Figure 5.2: Isoforms of the leptin receptor derived from differential splicing (Adapted from Wolf *et al* 2002).



These receptors share an identical extracellular amino-terminal end, which binds leptin, but differ at their carboxy-terminal ends. Five of these isoforms contain transmembrane domains. The long form of the receptor (Ob-Rb), principally found in the hypothalamus, is classically associated with signal transduction. The short form (Ob-Ra), expressed in peripheral tissues, is much more abundant.
Ob-Re, which lacks both transmembrane and intracellular domains, is a circulating leptin receptor of unknown significance. – Intracellular domain; - Transmembrane domain.

Figure 5.3: Summary of the numerous pathways in which leptin has been shown to be involved (From Fruhbeck 2006).



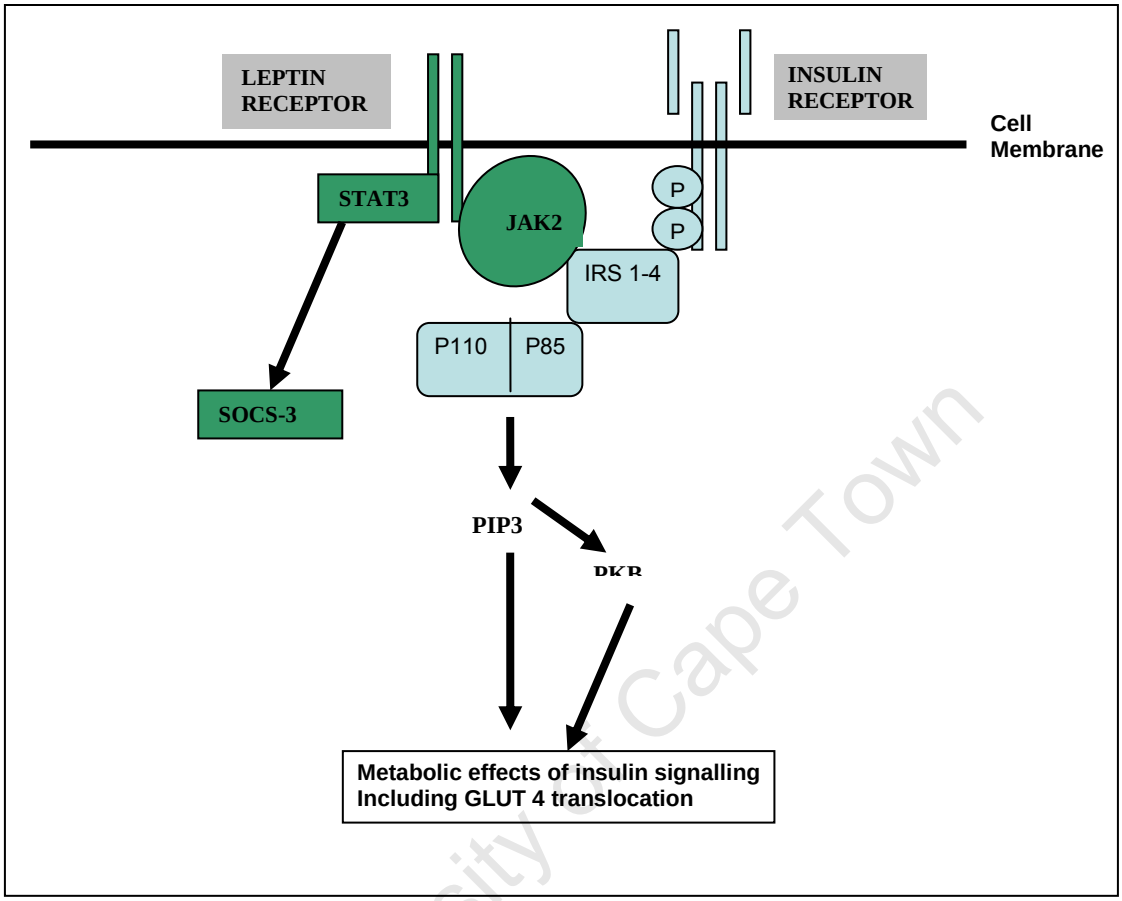
The JAK/ STAT pathway was the first signalling mechanism associated with the leptin receptor (Vaisse *et al* 1996). Activation of this signalling pathway in the hypothalamus is initiated upon the conformational changes in the leptin receptor triggered by leptin binding. Intracellular JAK proteins (JAK2 and possibly JAK1) are associated with the binding motifs located in the proximal region of the leptin receptor and are subsequently activated by transphosphorylation. Activated JAK proteins, in turn, phosphorylate tyrosine residues of the receptor, providing docking sites for STAT proteins, which become tyrosine-phosphorylated by JAK.

Phosphorylated STAT molecules dimerize and translocate to the nucleus to modulate the transcription of target genes. *In vitro* studies have shown that leptin receptors signal through different STAT molecules, *in vivo* studies have demonstrated that, in the hypothalamus, this occurs specifically through activation of STAT3 (Bates *et al* 2003).

The STAT3 signalling by leptin receptors mediates the critical effects of leptin on food intake and body mass control. Also, leptin's effects on the suppressors of the cytokine signalling family (SOCS) represent a negative-feedback mechanism proposed to underlie the development of leptin resistance in relation to the hyperleptinaemia seen in the majority of obesity cases (Banks *et al* 2000). SOCS proteins negatively regulate the JAK/STAT pathway, either by directly blocking JAKs or through inhibition of further STAT phosphorylation (Bjorbaek *et al* 1998 a).

Several other intracellular signalling mechanisms have been associated with the leptin receptor. For instance, by engaging JAK2, the leptin receptor is able to stimulate insulin receptor substrate (IRS)-2, which, in turn, activates phosphoinositol- 3 kinase (PI3-K) through an association to its regulatory subunit (Niswender *et al* 2001) (Figure 5.4). Most insulin-dependent actions involve PI3K activation, making this a relevant point of cross-talk between the insulin and leptin signalling pathways (Fruhbeck and Salvador 2000a). PI3K activity is also required for leptin-mediated sympathetic nerve activation (Rahmouni *et al* 2003).

Figure 5.4: Cross-talk of leptin signalling with insulin signalling pathways (Adapted from Fruhbeck 2006).



Leptin shares with other cytokines, growth factors and stressors the ability to activate the stress-activated protein kinase, JNK (c-Jun N-terminal kinase). Through this mechanism, leptin reportedly enhances TNFα production via p38 and JNK MAPK (Shen *et al* 2005). In vascular smooth muscle cells, leptin induces hypertrophy via p38 MAPK (Shin *et al* 2005), indicating the potential relevant role of this hormone in cardiovascular physiology and an impact on vascular remodelling (Fruhbeck and Salvador 2000b).

Although a definitive picture of leptin signal transduction, including upstream activators and downstream targets of the p38 and JNK MAPK pathways, is yet to be completely understood,

the regulation of NF-κB appears also to be a likely downstream target of leptin, since this essential transcription factor is known to play a pivotal role in the transcriptional regulation of pro-inflammatory cytokines such as TNFα and IL-1β.

5.4 LEPTIN AND THE CARDIOVASCULAR SYSTEM

5.4.1 LEPTIN, VASCULAR FUNCTION AND BLOOD PRESSURE

Like other metabolic hormones such as insulin, leptin possesses potent vascular effects and participates in the regulation of sympathetic tone and arterial blood pressure (Haynes *et al* 1997). The regulatory effects on vascular tone and blood pressure have been experimentally demonstrated. Intracerebroventricular administration of leptin was found to elicit an increase in arterial blood pressure (Matsumura *et al* 2000), consistent with its ability to activate the sympathetic nervous system. Also, chronic intravenous infusion of leptin has significantly enhanced arterial blood pressure associated with an elevated circulating plasma leptin level (Shirasaka *et al* 2003).

Leptin also has direct peripheral vascular actions through some vasoactive mediators such as NO and endothelin-1 (ET-1) as it has been shown directly to induce relaxation of the blood vessels through NO-dependent as well as NO-independent mechanisms (Kimura *et al* 2000). Leptin has been shown to stimulate endothelial NO synthesis, upregulate ET-1 production and promote accumulation of reactive oxygen species (ROS) in human umbilical vein endothelial cells (Lembo *et al* 2000; Quehenberger *et al* 2002).

As evidence suggests an association between sympathetic activity and plasma leptin levels, factors like hyperinsulinaemia have also been suggested to be responsible for the enhanced sympathetic activation and blood pressure rise in obesity.

5.4.2 LEPTIN, HEART RATE AND CARDIAC FUNCTION

Acute leptin infusions have been shown to fail to significantly alter the heart rate whereas chronic infusions have elicited a significant increase in heart rate along with an increase in sympathetic nervous activity (Shek *et al* 1998). Also, a positive correlation between hyperleptinaemia and tachycardia has been confirmed in mildly obese or mildly hypertensive human subjects (Narkiewicz *et al* 1999). While this increase in heart rate may enhance cardiac output and provide short-term benefits, sustained tachycardia may cause cardiac hypertrophy and lead to heart failure. Patients with advanced congestive heart failure have also exhibited elevated plasma levels of leptin and its soluble receptor, indicating that leptin may participate in the catabolic state leading to the development of cardiac cachexia (Schulze *et al* 2003).

5.4.3 LEPTIN AND ATHEROSCLEROSIS

Hyperleptinaemia is believed to be associated with lower arterial distensibility, a circulatory function relevant to the atherosclerotic process (Singhal *et al* 2002). Leptin promotes angiogenesis and potentiates the pro-thrombotic aggregation of platelets through a novel leptin receptor-dependent mechanism (Konstantinides *et al* 2001). The mechanisms of leptin induced atherosclerosis include: endothelial dysfunction through large amounts of NO generated from inducible nitric oxide synthase (Naseem 2005), abnormal lipid metabolism in the vessel wall (Maingrette and Renier 2003), induction of a proinflammatory state (La Cava *et al* 2004), increased reactive oxygen species thereby causing oxidative stress in the vessel wall (Bouloumie *et al* 1999), increased proliferation and migration of vascular smooth muscle cells (Li *et al* 2005) and increased platelet aggregation and abnormal haemostasis (Konstantinides *et al* 2001).

Designed to examine the effectiveness of pravastatin in primary prevention of ischemic heart disease in men with moderate hypercholesterolemia, the West of Scotland Coronary Prevention Study (WOSCOPS) is one among many clinical studies that has shown convincing evidence of the proatherogenic role of leptin in humans. This study demonstrated that elevated plasma leptin predicted acute cardiovascular events (combined acute myocardial infarction, need for revascularization and mortality) during a 5-year follow-up period (Wallace *et al* 2001).

5.5 LEPTIN AND RENAL DISEASE

Accumulating data suggest that leptin may exert direct and indirect effects on the kidney that may contribute to deterioration in renal function (Stenvinkel *et al* 1999). In this section, the importance of leptin as a progression factor in renal disease (from the review of available reports on this subject) will be explored.

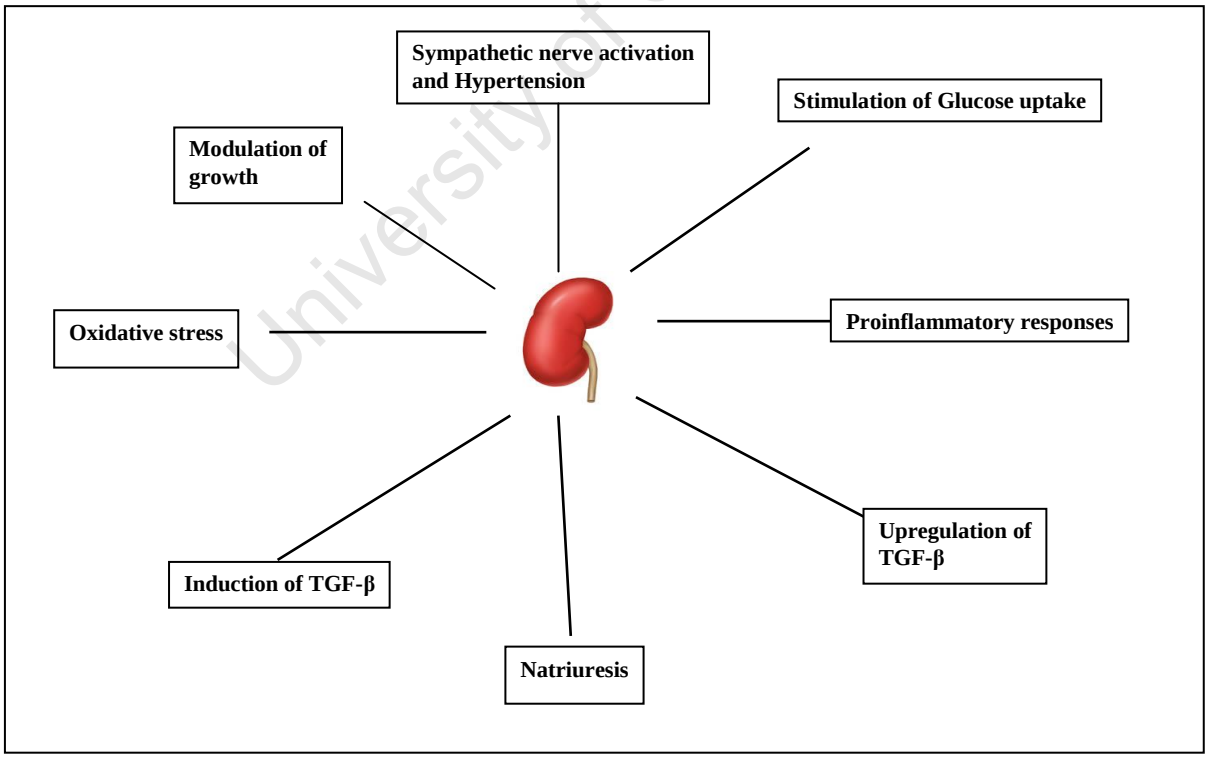
5.5.1 RENAL CLEARANCE OF LEPTIN

Leptin is principally cleared by the kidney (Nordfors *et al* 1998) consequently, plasma leptin concentrations are increased in patients with end-stage renal disease or those undergoing hemodialysis (Sharma *et al* 1997). A number of studies have investigated a relationship between leptin concentration and obesity in chronic renal failure (Stenvinkel *et al* 1999). Animal studies have shown that renal elimination of leptin occurs by the uptake of leptin into renal tissue, rather than by glomerular filtration (Zeng *et al* 1997).

5.5.2 LEPTIN'S ACTIONS IN THE KIDNEY

The renal effects of leptin are summarised in the figure 5.5. Leptin mediates sympathetic nerve activation and infusions of leptin into rats have been shown to increase renal sympathetic nervous activity (Haynes *et al* 1997). In the transgenic skinny mouse, which overexpresses leptin, systolic blood pressure and urinary catecholamine excretion are elevated (Aizawa-Abe *et al* 2000). Two proopiomelanocortin products, α-melanocyte-stimulating hormone (α-MSH) and β-endorphin, have been suggested as the possible mediators of these effects of leptin on sympathetic activity and mean arterial pressure (Dunbar and Lu 1999).

Figure 5.5. Summary of the effects of leptin that may contribute to pathological changes in the kidney (Adapted from Wolf *et al* 2002).



Transforming growth factor-beta (TGF-β) is a recognised mediator of fibrogenesis in various renal diseases and leptin has been shown to stimulate the proliferation of cultured glomerular endothelial cells and to induce mRNA expression and protein secretion of transforming growth factor-β1 (TGF-β1) in these cells (Wolf *et al* 1999).

Short-term infusion of leptin (72 hours) induced glomerular TGF-β1 expression and also increased the total number of proliferating cells detected by proliferating cell nuclear antigen staining. However, long-term infusion of leptin (3 weeks) enhanced glomerular expression of type IV collagen. However, in mesangial cells, leptin failed to stimulate TGF-β1 synthesis, but dose-dependently increased mRNA levels in TGF-β type II receptors in *db/db*-derived mesangial cells as well as cellular glucose uptake, augmented type 1 collagen mRNA and protein production (Han *et al* 2001). This suggests that the leptin system may cross-talk between glomerular endothelial and mesangial cells. (See figure 4.3) Activation of this paracrine TGF-β system by leptin eventually contributes to extracellular matrix deposition, glomerulosclerosis, and proteinuria (Ballermann 1999).

5.5.3 LEPTIN AND THE KIDNEY IN SYSTEMIC DISEASES

An increased incidence of glomerulosclerosis has been described in patients with massive obesity (Kasiske and Crosson 1986). Because obese individuals show a marked increase in serum leptin concentrations, the hormone may contribute to the development of glomerulosclerosis seen in this population. This effect may be exaggerated by the loss of functioning nephrons. Praga *et al* (2000) reported that obese patients are at high risk for developing proteinuria and chronic renal failure after unilateral nephrectomy (Praga *et al* 2000).

It has also been suggested that the pathophysiological effects of leptin may be more relevant in patients with type 2 diabetes who have high serum leptin concentrations (Widjaja *et al* 1997). A fibrogenic role for leptin in diabetic nephropathy remains to be established; however, clinical clues are suggestive: massively obese patients with high serum leptin levels tend to develop focal glomerulosclerosis whereas patients with type 2 diabetes and hyperinsulinemia, who tend to be obese, exhibit high serum leptin levels (Widjaja *et al* 1997).

Leptin levels are increased in patients with type 2 diabetes with microalbuminuria or macroalbuminuria (Fruehwald-Schultes *et al* 1999) and urine leptin concentrations in Pima Indians have positively correlated with urinary albumin-creatinine ratios and inversely correlated with glomerular filtration rates (Wilson *et al* 1998). Increased serum leptin levels also correlated with increased urine albumin excretion in women with type 1 diabetes (Rudberg and Persson 1998).

These observations raise the possibility that leptin may cooperate with other mediators to promote increased ECM production in some forms of renal disease, such as diabetic glomerulopathy.

CHAPTER 6

GENETICS: OBESITY, KIDNEY DISEASE AND THE METABOLIC SYNDROME

6.1 INTRODUCTION

The ability to pass on specific traits to offspring has allowed humans to adapt through genetic selection to changing situations (Fauci *et al* 2001). Hence, an individual's genotype is able to influence both cell development and functions throughout life and the phenotype results from the interaction of the genotype and environmental influences. However, the process of associating a gene with a phenotype is made difficult by the low density of functionally coded DNA and by multiple interactions among genes and lifestyle factors (Shuldiner and Munir 2003).

As CVDs generally result from interactions of multiple lifestyle factors, it is assumed that to find specific genes that account primarily for specific CVD will be a very difficult task. However, a number of studies either using the candidate gene approach or the genome wide scan have reported genes related to obesity, the MS and kidney diseases. This chapter gives an overview of such genes which have been reported in the literature to be associated with these conditions. In the context of this thesis I will use the term polymorphism (and single nucleotide polymorphism (SNP)) as referring to an inheritable change in a single nucleotide of a specific sequence of genomic DNA among individuals that occurs in more than 1% of the population. Generally, a SNP can occur through inversion, deletion, repetition, insertion or translocation within chromosomal segments (Fauci et al 2001).

6.2 THE GENETICS OF OBESITY

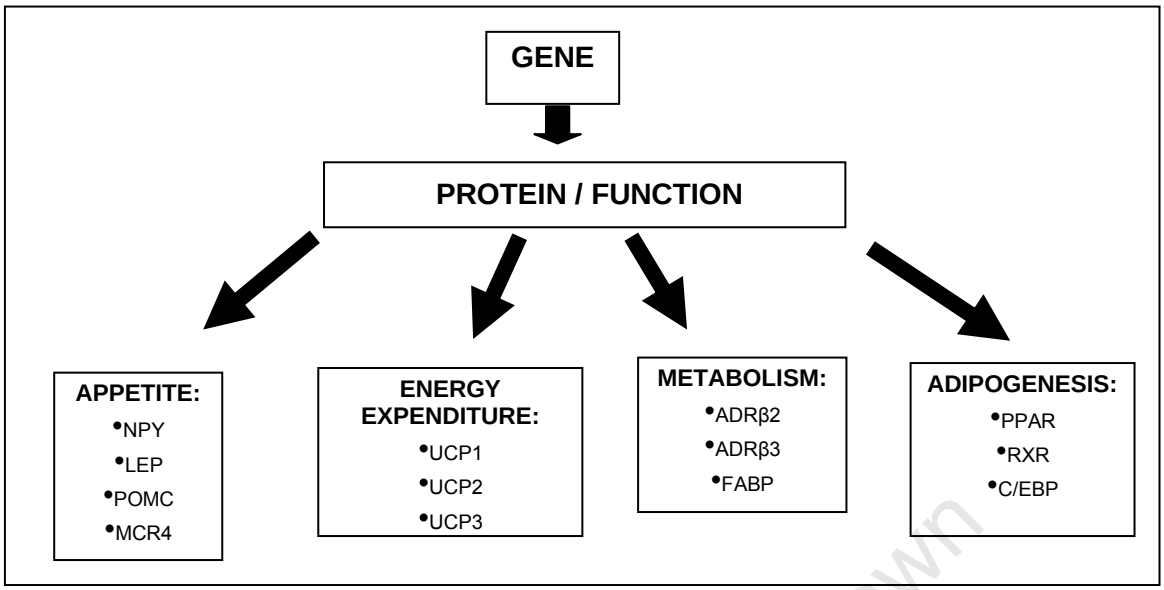
Obesity involves environmental and genetic factors. The environmental factors which influence obesity have been discussed in chapter 2. The role of genetic factors in obesity mostly comes from the commonly observed coexistence of several obese members within a family. In some families with obese parents, it has been observed that the risk of excessive weight gain in children of such families is increased two- to three-fold for moderate obesity and up to eight times for severe obesity (Bouchard 2001). Also, twin and adoption studies substantiate a role for genetics in obesity. Allison et al. (1996) have reported estimates of heritability (h^2) of obesity to be 0.65, 0.75 and 0.85 in 17 Finnish, 10 Japanese and 26 American twin pairs respectively. Figure 6.1 shows some genes involved in weight homeostasis categorised by their mechanism of obesity.

6.2.1 MONOGENIC FORMS OF OBESITY

The mutations in genes that encode proteins with likely roles in appetite regulation are responsible for Mendelian disorders in which obesity is the most obvious phenotype.

Rare monogenic recessive forms of human obesity such as those caused by mutations in the genes that encode leptin (LEP) (Montague *et al* 1997), leptin receptor (LEPR) (Clement *et al* 1998), and proopiomelanocortin (POMC) (Krude *et al* 1998) all of which result in a phenotype of excessive energy intake relative to energy expenditure have been reported. The regulatory role of molecules like the melanocortin 4 receptor (MC4R) and the agouti related peptide (AGRP) in monogenic forms of obesity have also been established through genetic manipulation (Ollmann *et al* 1997).

FIGURE 6.1: Some genes involved in body weight homeostasis categorised by processes.



NPY – Neuropeptide Y, LEP – Leptin, POMC – Pro-opiomelanocortin, MCR4 – Melanocortin receptor type 4, UCP – Uncoupling protein, ADRβ – Adrenergic receptor beta, FABP – Fatty acid binding protein, PPAR – Perioxisome proliferator-activator receptor, RXR – Retinoid X receptor, C/EBP – CCAAT/ Enhancer binding protein.

6.2.2 SYNDROMIC OBESITY

Syndromic obesity refers to Mendelian disorders in which patients are clinically obese and additionally have characteristics such as mental retardation, dysmorphic features, and organ-specific developmental abnormalities (Farooqi and O'Rahilly. 2005). These syndromes arise from discrete genetic defects or chromosomal abnormalities, and can be either autosomal or X-linked disorders. Although these syndromes have been well characterised clinically, analysis of their genetic components have shown that multiple genes within a biological pathway may produce similar phenotypes (Chung and Leibel. 2005). The most common disorders known are Prader-Willi syndrome, Bardet-Biedl syndrome, and Alstrom syndrome, but many others have been reported as well.

6.2.3 POLYGENIC OBESITY

Polygenic obesity refers to the common variant of obesity. This form of obesity has a substantial contribution from environmental / lifestyle factors as well as genetic factors involving several genes, each contributing a small part to the overall obesity phenotype. Studying the genetic basis of common obesity usually involves the analysis of variations in genomic DNA situated within or near candidate genes. The aim of genetic studies, whether they are carried out in family members or unrelated subjects and depending on the statistical methods employed (linkage family studies, association studies in unrelated obese subjects), is to establish whether an association exists between a particular variant or 'allele' of a gene and obesity phenotype which is often defined as BMI, WHR or WC (Hebebrand *et al* 2003). Two different approaches to elucidating the genetic basis of disease are described below.

6.2.3.1 The genome regions linked to obesity

This method employs the systematic examination of all the chromosomes in the families of obese subjects using highly polymorphic markers to detect increased allelic sharing in obese sibpairs. This task is performed without preconceptions about the functions of the genes and aims to identify known or uncharacterised genes predisposing to obesity. This method has been applied to different patient cohorts worldwide and has brought to light several chromosomal locations linked to obesity including those on chromosomes 2, 3, 5, 6, 7, 10, 11, 17 and 20 (Loos and Bouchard 2003).

6.2.3.2 Candidate genes

This approach entails the identification of good candidate genes for the disease according to the properties of those genes. The candidate gene approach in the genetic analysis of obese populations in different countries has so far not revealed the predominant and unambiguous

role of such candidate genes in the pathogenesis of common obesity. The reasons for this includes the difficulty of replicating most association and linkage results performed in different populations, usually through the lack of statistical power to detect modest effect, lack of control over type-1 error rate (a false positive error - the proportion of negative instances that were erroneously reported as being positive), complex population stratification and over-interpretation of marginal data. Other reasons include non-genetic factors like environmental or cultural factors that differ between populations and are known to strongly influence the development of obesity.

The choice of a candidate gene in obesity research is usually based on several arguments including the physiological role of its encoded protein in the mechanism of obesity, its chromosomal location in a region linked to obesity in human or animal models, the phenotypic consequences of its genetic manipulation in rodent models and eventually the in vitro functional characteristics of gene mutations or variations studied. The obesity gene map published each year extensively summarizes all the genes and variants screened (Perusse *et al* 2005). Table 6.1 shows a list of genes commonly associated with the obesity phenotype in humans.

Table 6.1: Examples of genes frequently associated with obesity phenotypes in humans

Genes (HGNC Code)	Locus	Human Obesity Locus
Food intake:		
LEP	7q31	Yes
LEPR	1p31	Yes
AGRP	16q22	Yes
Energy metabolism:		
UCP 1	4q28-31	No
UCP 2	11q13	Yes
UCP 3	11q13	Yes
ADRβ3	9p12	Yes
GNB3	12p13.3	Yes
Adipose tissue metabolism:		
ACDC	3q27	Yes
PPARG	3p25	No
TNF-α	6p21.3	Yes
ADRβ2	5q31-32	Yes
IL-6	7p21	No
LIPE	19q13.2	Yes
GR	5q31	Yes
Lipid and glucose metabolism:		
INS	11p15.5	Yes
LDLR	19p13	Yes

HGNC - Hugo Gene Nomenclature Committee

LEP – Leptin, LEPR – Leptin receptor, AGRP – Agouti related peptide, UCP –Uncoupling protein, ADRβ – Adrenergic receptor beta, GNB3 – G-protein beta 3, ACDC – Adiponectin, PPARG – Peroxisome proliferator activator receptor gamma, TNF-α – Tumour necrosis factor alpha, IL-6, Interleukin 6, LIPE – Lipase, hormone sensitive, GR – Glucocorticoid receptor, INS – Insulin, LDLR – Low density lipoprotein receptor.

6.3 THE OBESITY GENE (LEP)

6.3.1 DESCRIPTION

The leptin gene (LEP) is the human homolog of the obesity gene (ob) in the mouse 'obese' phenotype. The ob gene of the mouse was isolated in 1994 by positional cloning by Zhang *et al* and the group also cloned and sequenced its human homolog (Zhang *et al* 1994). The gene encodes a 4.5-kb adipose tissue mRNA with a highly conserved 167-amino acid open reading frame. The predicted amino acid sequence is 84% identical between human and mouse and has features of a secreted protein. In 1991, Friedman *et al* suggested that the human ob homolog maps to 7q31 (Friedman *et al* 1991). In the perspective of this thesis, LEP will be used as referring to the obesity gene.

In 1995, Green *et al* mapped the human LEP gene on a YAC contig (a contig is a set of overlapping DNA segments derived from a single genetic source) from 7q31.3 that contained sequence tagged sites corresponding to microsatellite-type genetic markers. Because of their close physical proximity to the human LEP gene, these genetic markers represented valuable tools for analyzing families with evidence of hereditary obesity and for investigating the possible association between LEP mutations and human obesity.

6.3.2 GENE STRUCTURE

The LEP gene spans approximately 20,000 bases and contains 3 exons separated by 2 introns (Isse *et al* 1995). The gene's 3 exons cover approximately 15 kb of genomic DNA. The entire coding region is contained in exons 2 and 3 separated by a 2-kb intron. The first small 30-bp untranslated exon is located more than 10.5 kb upstream of the initiator ATG codon (Miller *et al* 1996). The first intron, approximately 10.6 kb, is in the 5-prime untranslated region, 29 bp upstream of the ATG start codon. The second intron of 2.3 kb is located at glutamine +49. By rapid amplification of 5-prime cDNA ends, the transcription initiation sites were mapped 54

to 57 bp upstream of the ATG start codon (Isse *et al* 1995). The gene structure and common polymorphisms of the LEP gene are shown in figure 6.2

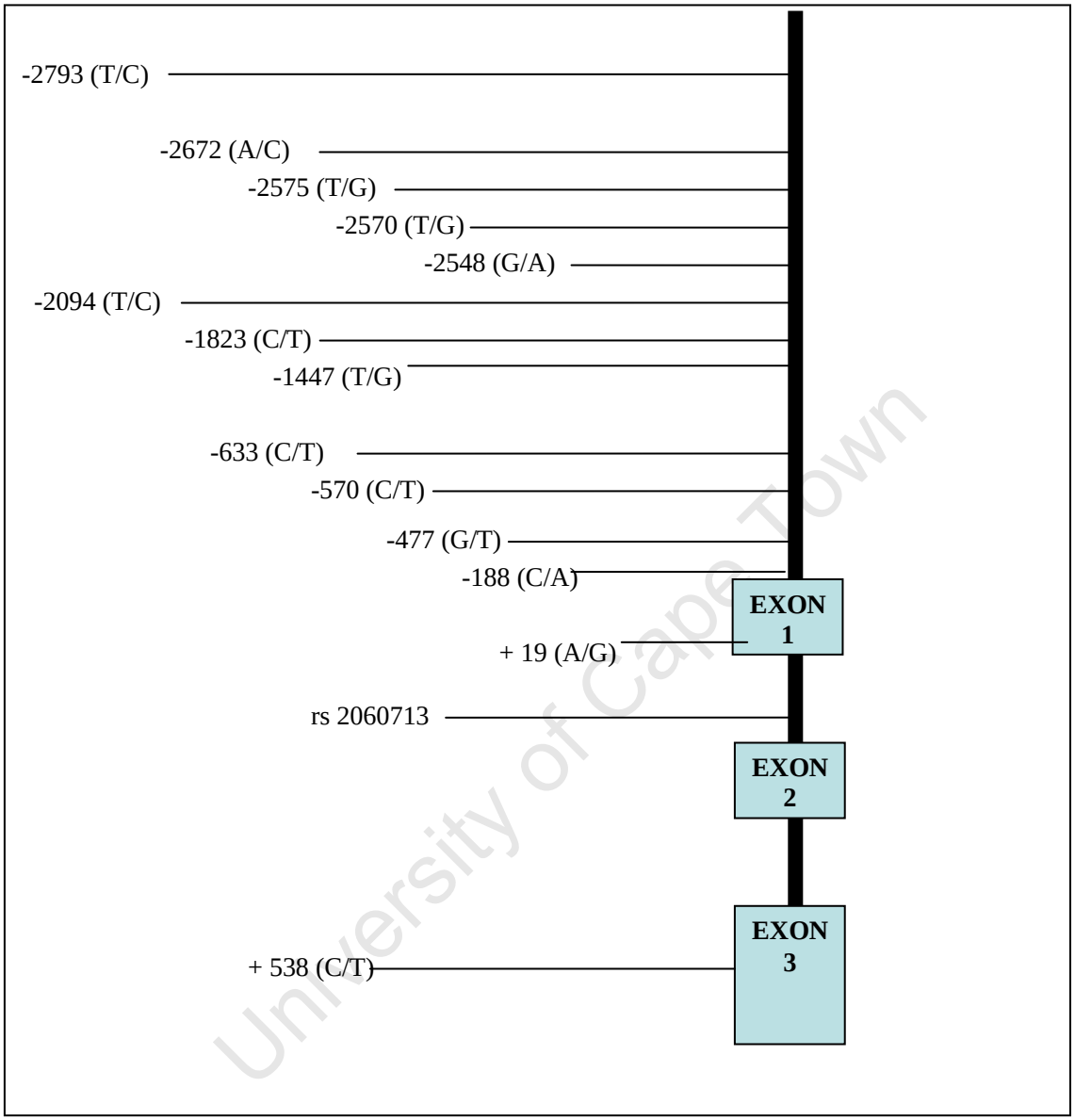
6.3.4 GENE FUNCTION

(The functions of the gene via its protein leptin have been discussed in the previous chapter).

6.3.5 LINKAGE TO OBESITY-RELATED TRAITS

Duggirala *et al* (1996) tested for linkage between various obesity-related traits plus associated metabolic traits in 15 markers on human chromosome 7 by using sibship data obtained from 32 Mexican American pedigrees. They found evidence for linkage between markers in the LEP gene region and obesity phenotype measured as extremity skin folds (LOD = 3.1) (LOD is the total relative probability, expressed on a logarithmic scale, that a linkage relationship exists among selected loci and is an acronym for "logarithmic odds"). A putative susceptibility locus linked to the marker D7S514 explained 56% of the total phenotypic variation in extremity skin folds. Weaker evidence for linkage to several other obesity-related traits (e.g., WC, BMI, fat mass by bioimpedance, etc.) was observed for a genetic location approximately 15 cM telomeric to LEP (Duggirala *et al* 1996).

Figure 6.2: Commonly reported polymorphisms of the LEP gene (Adapted from Li et al 1999)



6.3.6 MOLECULAR GENETIC STUDIES RELATED TO THE LEP GENE

The LEP gene expression in abdominal subcutaneous adipocytes from lean and obese humans has been studied by Considine *et al* (1995) and they detected no difference in the sequence of an RT-PCR product of the coding region from 5 lean and 5 obese subjects. Later, Hager *et al* (1998) screened patients with morbid obesity for mutations in the leptin gene and for association with a polymorphism in the 5-prime untranslated region of the gene. Although no polymorphisms were detected in the coding region of the gene, a single A-to-G transition was found in nucleotide 26 of the untranslated first exon. In metabolic studies, they showed that patients homozygous for the G allele of the exon 1 variant had significantly lower fasting leptin levels compared to subjects being either heterozygous (AG) or homozygous for the A allele despite a similar BMI ($p = 0.01$).

Eight genetic variants were later identified (C2549A, T2437G, C1887T, C1823T, G1387A, C633T, C188A, G19A) in the 5-prime region of the LEP gene and one of the mutations (2548G-A), wrongly designated 2549C-A at that time, was found to be associated with a difference in BMI reduction following a low calorie diet in overweight women (Mammes *et al* 1998). The genotype and allele frequencies of this SNP were significantly different between groups, with the G allele being more frequent in the overweight subjects ($p < 0.01$). In men, carriers of this allele had lower leptin concentrations adjusted for fat mass. The results indicated that variations at the leptin locus are associated with common obesity phenotypes, and not only with extreme obesity or the rare Mendelian obesity syndromes (Mammes *et al* 2000).

Karvonen and his group (1998), studying the LEP gene for variants by screening both its putative promoter and its coding region in 200 obese Finnish subjects found a 144G-A transition in codon 48 and a 328G-A transition in codon 110 in 2 obese subjects, both of whom had very low serum leptin levels. A rare silent polymorphism (538C-T) was also reported 33 bp downstream of the translation stop codon (TGA), and a common polymorphism (19A-G) was identified in the untranslated exon 1. This polymorphism was not associated with obesity and the allele frequencies were similar between 64 normal-weight and 141 obese Finns. The authors concluded that there is no common LEP gene variation associated with obesity in that population. Lucatoni *et al* (2000) also found no significant correlations between the A19G polymorphism and serum leptin levels and other metabolic parameters they considered (Lucatoni *et al* 2000).

The genetic association of the leptin gene polymorphism with obesity, insulin resistance, and hypertension was examined by Shintani *et al* (2002). They found no significant difference in BMI with the different genotypes in both hypertensives and controls. However, they reported a higher frequency of class I genotype in the hypertensives and concluded that the leptin gene polymorphism was associated with hypertension independent of obesity.

There was only one study where polymorphism of the LEP gene was reported to be associated with specific cardiovascular markers. The impact of this gene on blood pressure, BMI and subclinical atherosclerosis was assessed and it was found that the rare T allele at the C538T polymorphism substantially influenced pulse pressure and carotid intima-medial thickness, but did not appear to exert this effect through actions on plasma leptin level or BMI. This suggested that an autocrine or paracrine effect in vascular tissue may be an important physiologic function of leptin (Gaukrodger *et al* 2005).

6.4 GENETICS OF KIDNEY DISEASE

6.4.1 EVIDENCE FOR A GENETIC COMPONENT OF CKD/ESRD

ESRD is widely held to be a complex genetic trait with significant genetic heterogeneity, and gene–gene and gene–environment interactions. This hypothesis is supported by extensive evidence that ESRD in the black population has a strong genetic component (Freedman 2002). Black Americans with close relatives having ESRD are at markedly increased risk for developing future ESRD (Freedman *et al* 1993). Evidence suggests that renal disease in the general population has a genetic component. Studies that have shown such evidence take the form of evaluating familial aggregation of CKD and or ESRD. The first description of familial aggregation in renal disease was carried out in Caucasian type 1 diabetic (T1DM) families in which individuals who had undergone kidney transplantation were compared to families who contained diabetes-affected individuals without substantial renal disease (Seaquist *et al* 1989). In this study, significant renal disease was reported in the diabetic siblings of probands receiving renal transplants compared to diabetic siblings of probands without nephropathy. Familial clustering of renal disease has been described in patients with type 2 diabetes (T2DM) from different populations (Bowden 2003).

6.4.2 CKD AS A COMPLEX GENETIC TRAIT

Multiple lines of evidence indicate that susceptibility to the development and progression of kidney disease is genetically determined (Bowden 2003). Three main sources of evidence for a genetic contribution to kidney disease risk were identified as: (1) diabetic and non-diabetic ESRD clustering in families; (2) after controlling for socioeconomic status in geographically dispersed populations, incidence of kidney disease in ethnic minorities in the US and aboriginal peoples in Australia is increased compared to patients of European descent; and (3) segregation analyses suggesting a major genetic effect contributing to diabetic

nephropathy and urinary albumin excretion in diabetic Pima Indians and Caucasians, respectively (Bowden 2003).

Genetic pathways regulating susceptibility to common forms of CKD are difficult to explain. Since CKD is a heterogeneous entity, no one pathophysiologic mechanism leads to kidney disease, but rather multiple processes that may include immunologic, metabolic, hemodynamic, ischemic, toxic, or anatomic, work together to cause kidney injury. Often at diagnosis, 60% to 70% of nephrons have already been destroyed, further confounding the identification of initiating CKD mechanisms. Hence, from a genetic perspective, common causes of CKD represent complex traits. There is no simple or known link between the clinical manifestations of the disease phenotypes and genetic mutations and with no obvious Mendelian inheritance patterns, mutations causing monogenic kidney diseases do not appropriately model the genetic mechanisms of more common kidney diseases such as diabetic nephropathy.

Genetic mutations responsible for Mendelian syndromes result in loss of protein function and early onset clinical phenotypes. In contrast, genes that regulate common CKD are better described as “liability alleles”, whose genetic variations have modest impact on protein function. Unlike most mutations for Mendelian kidney diseases, liability alleles only result in affection when a biological threshold is exceeded. In other words, CKD “liability alleles” each have a small individual effect but can sum together to result in disease. Further complicating genetic analyses, CKD risk is modified by environmental factors, especially diabetes (i.e. glucose control) and hypertension (i.e. blood pressure control), gene-gene interactions and gene-environment interactions (Padiyar and Sedor 2005).

6.4.3 THE SEARCH FOR RENAL DISEASE GENES

With the wide acceptance that inheritance contributes to renal disease susceptibility, investigators have applied two main methods, using different study designs to identify ESRD genes.

6.4.3.1 Candidate genes

Using this method, specific genes are selected for analysis based on a function that could be closely related renal disease or through direct evidence that the gene product (protein) is associated with renal pathology. Often, these studies are done as case-control studies in which the allele frequencies in renal disease patients are compared with allele frequencies in patients without renal disease. Table 6.2 summarises some candidate genes that have shown association with renal disease, the populations they were studied in and the mechanism of renal disease.

6.4.3.2 Genome wide screen

This approach is more difficult than the candidate gene approach but has the advantage of being able to locate new (novel) genes. This approach gives a comprehensive map of inheritance of all parts of the human genome in families expressing a specific trait like ESRD. Since the Phoenix group first reported their finding in T2DM Pima Indians of kidney disease linkage to chromosome 7 (Imperatore *et al* 1998), a number of other genome wide screens for kidney disease have been carried out.

Table 6.2: Candidate genes reported to be associated with kidney disease.

GENE (HGNC CODE)	REFERENCE	POPULATION	MECHANISM
Renin-Angiotensin System (RAS)			Effect on tissue haemodynamics and glomerular cell function
- ACE	Yoshida *et al* 1996	DM1, DM2 Nephropathy	
- AGT	Rogus *et al* 1998	DM1 Nephropathy	
Growth factors and cytokines			Mesangial cell proliferation, ECM production and role in renal hypertrophy
- IL-1	Loughrey *et al* 1998		
- IL1-RN	Shu *et al* 2000	DM1, DM2 Nephropathy	
- TNF-α	Shu *et al* 2000		
- TGF-β	Reeves and Andreoli. 2000		
Kallikrein-kinin system			
- KLKB1	Yu *et al* 1998	Non-DM renal disease	
- KLK1 Promoter	Yu *et al* 1998	Non-DM renal disease	Inflammatory process
- BDKRB1	Zychma *et al* 1999	ESRD	
Vasoactive mediators			
- EDN	Freedman *et al* 2000		Regulation of vascular tone and Homocysteine metabolism
- NOS	Yokoyama *et al* 1998	Non-DM renal disease	
- MTHFR	Kimura *et al* 2000		

HGNC - Hugo Gene Nomenclature Committee

DM1 – Type 1 diabetes, DM2 – Type 2 diabetes, ECM – Extracellualr matrix

ACE – Angiotensin converting enzyme, AGT – Angiotensinogen, IL-1 – Interleukin 1, IL1-RN – Interleukin 1receptor antagonist, TNF-α – Tumour necrosis factor alpha, TGF-β – Transforming growth factor beta, KLKB1 – Kallikrein B, KLK Promoter – Kallikrein promoter, BDKRB1 – Bradykinin receptor B, EDN – Endothelin, NOS – Nitric oxide synthase, MTHFR – Methylenetetrahydrofolate reductase.

Freedman *et al* (2003) performed a genome-wide scan to assess for loci contributing to urine albumin-to-creatinine ratio (ACR) in participants enrolled in the Hypertension Genetic Epidemiology Network (HyperGEN study) (Freedman *et al* 2003). Linkage was tested between 387 markers spanning the genome at an average interval of 9.32 cM. The genome-wide scan revealed a maximum LOD score of 2.73 on chromosome 19 and a LOD score of 2.0 on chromosome 12. Their findings demonstrate the marked heritability of urine ACR in families enriched for the presence of members with essential hypertension and suggest that a gene(s) associated with urinary ACR may be present on human chromosomes 19 and 12. Other genome wide studies are summarised in table 6.3.

There has been little consistency among these studies, probably reflecting differences in phenotypes and population groups studied. A region that has shown some consistency across several studies is the 7p region linked to GFR in type 2 diabetes studies (Placha *et al* 2005). A genome-wide panel of 372 autosomal short tandem repeat markers was typed by Chen et al (Chen et al. 2007) in 691 West Africans with type 2 diabetes by using a multipoint variance components linkage on 3 phenotypes of renal disease. Linkage to creatinine clearance was observed on chromosomes 16 (LOD score = 3.56, p=0.0001), 17 (LOD score = 2.08, p = 0.0018), and 7 (LOD score = 1.84, p=0.0022). This is highly supportive of the leptin gene on chromosome 7 as a potential link to renal disease, especially in black Africans.

Table 6.3: Genome wide studies for kidney disease in different populations.

Author	Population	Chromosomes	Renal Marker
DeWan *et al* 2001	Hypertension, African American, Caucasians	3	Creatinine clearance
Freedman *et al* 2002	T2DM, ESRD, African American	10	GFR
Freedman *et al* 2003	Hypertension, African American	12, 19	Urine ACR
Freedman *et al* 2004	Non-diabetic, ESRD, African American	1, 9, 13	GFR
Bowden *et al* 2004	T2DM	3, 7, 18	GFR
Sale *et al* 2004	T2DM, African American	6, 7, 18	Creatinine, urine ACR
Fox *et al* 2004	Framingham	3, 4	Creatinine, GFR, creatinine clearance
Freedman *et al* 2005	All cause ESRD, African American	1, 4, 9, 13	GFR
Chen *et al* 2007	T2DM, West Africans	3, 6, 7, 8, 10, 16, 17	Creatinine, creatinine clearance, GFR

T2DM – Type 2 diabetes mellitus

6.5 GENETICS OF THE METABOLIC SYNDROME

Family studies have clearly indicated a heritable component to MS; however several association studies have yielded a large number of MS-associated loci without subsequent identification of aetiological genes. To date, existing approaches have in general failed to identify genes underlying complex diseases or traits such as MS, as they often present with a wide range of phenotypes and generally involve multiple aetiological mechanisms and contributing genes. The contribution of each of several causative genes is likely to be small and only the joint effect of several susceptibility genes, often in concert with environmental factors, would lead to the complex disease state (Glazier *et al* 2002).

Some studies have suggested that the components of the MS may share genetic determinants. For instance, results from a Swedish study showed that all five principal components of the MS were influenced by a single latent genetic factor (Hong *et al* 1997). These studies indicate the presence of an underlying pleiotropic factor among the components of the MS.

6.5.1 CANDIDATE GENES FOR THE METABOLIC SYNDROME

The "thrifty gene hypothesis" proposes that efficient storage of energy could have been associated with survival advantage (Neel 1962) hence; efficient storage of energy must include storage of fat and weight gain. Obesity genes could therefore predispose to T2DM and therefore the MS. A number of candidate genes have been shown from both human and animal studies to be related to insulin resistance. Most of these genes are related to adipose tissue proteins or involved in glucose homeostasis.

6.5.1.1 Agouti, Pro-Opiomelanocortin (POMC), And Melanocortin Receptors (MCR)

The α-melanocyte-stimulating hormone (α-MSH) normally binds to the melanocortin 1 receptor (MCR1) in the skin to control pigmentation and to MCR4 in the hypothalamus to suppress appetite (Fan *et al* 1997). Also, the agouti protein normally antagonizes the effect of α-MSH on MCR1. However, when the mutated protein is ectopically expressed in the hypothalamus it antagonizes the effect of α-MSH on MCR4, resulting in uncontrolled appetite. In humans the counterpart of the agouti protein is the agouti-related peptide (AGTR; Ollman *et al* 1997). Mutations in the MCR4 gene have, in some families, been associated with morbid obesity (Vaisse *et al* 1998). The ligand for the melanocortin receptor α-MSH is, together with β-MSH, γ-MSH and ACTH, processed from pro-opiomelanocortin (POMC) by different peptidases.

There are several pieces of evidence to support a role of POMC in the pathogenesis of obesity. Neuropeptide Y (NPY), which normally stimulates appetite, downregulates the expression of POMC mRNA in the brain, whereas leptin, which suppresses appetite, up-regulates POMC expression. Linkage to the POMC region on chromosome 2p21 has been reported for leptin levels and resting metabolic rate (Comuzzie *et al* 1997). In one family, mutations in the POMC gene segregated with progressing obesity and ACTH deficiency (Krude *et al* 1998).

6.5.1.2 Beta-2 and Beta-3 Adrenergic Receptors (ADRβ2 and ADRβ3)

The β-3 adrenergic receptor (ADRβ3) is expressed in brown adipose tissue of rodents, is considered responsible for thermogenesis and has been shown to increase lipolysis in visceral fat from subjects with abdominal obesity (Lonnqvist *et al* 1995). Carriers of the ADRβ3

mutation have shown more abdominal obesity, higher insulin concentrations; more insulin resistance and higher blood pressure than individuals homozygous for the wild type (Widen *et al*. 1995) and *In vitro* studies have shown that the mutation may be associated with impairment in catecholamine-stimulated lipolysis (Hoffstedt *et al* 1999). A variant at codon 27 of the ADRβ2 gene has been associated with obesity and hypertension and a polymorphism of the ADRβ2 gene is in linkage disequilibrium with the codon 27 variant and is associated with obesity and type 2 diabetes in the Japanese (Large *et al* 1997; Yamada *et al* 1999).

6.5.1.3 Glycogen synthase

Impaired stimulation of glycogen synthesis by insulin is a hallmark of type 2 diabetes and IGT. The key enzyme of this pathway, glycogen synthase, could therefore be an important candidate for a genetic defect causing insulin resistance. The human glycogen synthase gene is assigned to chromosome 19q21 (Orho *et al* 1995) and a polymorphism of this gene has been associated with type 2 diabetes and insulin resistance, particularly impaired insulin-stimulated glycogen synthesis in skeletal muscle (Majer *et al* 1996).

6.5.1.4 Glycoprotein PC-1

The membrane glycoprotein PC-1 was isolated from a patient with extreme insulin resistance and found to inhibit insulin receptor tyrosine kinase activity (Maddux *et al* 1995). A variant in exon 4 of the PC-1 gene has been associated with insulin resistance and features of the MS (Pizzuti *et al* 1999).

6.5.1.5 Insulin receptor substrate -1 (IRS-1)

Two amino-acid polymorphisms were described in the IRS-1 gene (Almind *et al* 1993). These amino-acid substitutions, which were located close to the tyrosine phosphorylation site, were slightly more frequent in type 2 diabetic patients than in control subjects. In obese non-diabetic Danish subjects, the presence of the 972 polymorphism was associated with insulin resistance (Clausen *et al* 1995). The 972 variant of the IRS-1 gene seems to be predominantly increased in type 2 diabetic patients with the MS.

6.5.1.6 Leptin and the leptin receptor

A mutation in the LEP gene results in the complete absence of the protein in the *ob*/*ob* mouse and treatment of *ob*/*ob* mouse with leptin leads to marked weight loss (Pelleymounter *et al* 1995). However, in obese humans, an elevated rather than decreased level of leptin is seen and the leptin levels show a strong positive correlation with total fat mass. Two morbidly obese children from consanguine parents had very low circulating leptin levels due to a frameshift mutation involving a deletion of a single guanine nucleotide in codon 133 of the leptin gene (Montague *et al* 1997).

The leptin receptor belongs to the cytokine receptor family. The db mutation is due to an abnormally spliced leptin receptor with the mutant protein lacking the cytoplasmic region. It is suggested that the defect involves impaired leptin signalling in the hypothalamus (Lee *et al* 1996). Although the leptin signalling pathway has not yet been described in detail, it has been suggested that the leptin signal leads to inhibition of neuropeptide Y, which stimulates food intake and decreases thermogenesis (Stephens *et al* 1995).

6.5.1.7 Lipases

The breakdown of triglycerides is regulated by several lipases. A polymorphism in the hormone-sensitive lipase (HSL) gene was associated with type 2 diabetes which is seen in the MS (Klannemark *et al* 1998). Importantly, this variant was in a transmission disequilibrium test more often transmitted from heterozygous parents to abdominally obese offspring. A variant in exon 6 of the endothelial lipoprotein lipase (LPL) gene has been associated with high triglycerides, low HDL cholesterol and increased risk of CVD (Knudsen *et al* 1997).

6.5.1.8 Peroxisome proliferator-activated receptor Gamma (PPARγ)

There are three forms of PPAR receptors (α, β, γ) which induce transcription of a number of target genes in adipose tissue. The human PPARγ gene maps to chromosome 3p24, a region implemented in several genome-wide scans for type 2 diabetes. A rare Pro-115-Gln mutation in the *PPAR*γ gene that leads to inhibition of phosphorylation, resulting in increased adipocyte differentiation was described in four morbidly obese subjects (Ristow *et al* 1998). Another more common Pro-12-Ala variant has been associated with a high BMI (Ek *et al* 1999).

6.5.1.9 Tumour Necrosis Factor-Alpha (TNF-α)

TNF-α is a cytokine which is overexpressed in adipose and muscle tissue of obese animals and humans (Hotamisigil *et al* 1995). A positive correlation has also been demonstrated between the level of TNF-α mRNA in fat tissue and the level of hyperinsulinaemia, suggesting a role for this cytokine in the pathogenesis of insulin resistance (Hotamisligil *et al* 1993). Two polymorphisms (at positions 308 and 238) have been identified in the TNF-α gene. Although these polymorphisms appear to influence the host response to infections,

discrepant results have been obtained regarding a possible association with human obesity and insulin resistance (Walston *et al* 1999).

6.5.1.10 Uncoupling proteins (ucp)

The uncoupling proteins are proton-channel proteins on the inner mitochondrial membrane, and convert the electrochemical potential of the mitochondria into heat instead of ATP. They are responsible for thermogenesis in brown adipose tissue of rodents. While ucp1 is expressed almost exclusively in brown adipose tissue, ucp2 is expressed in most tissues including white adipose tissue. The *ucp3* gene is strongly expressed in skeletal muscle and so has been an attractive candidate gene for the MS. A polymorphism in the promoter region of the ucp1 gene on chromosome 4 has been associated with weight gain, especialy if the patient has the Trp64Arg mutation in the ADRβ3 gene (Clement *et al* 1996).

6.5.2 Genome-wide scan

Several genome-wide scans have been carried out with either obesity, type 2 diabetes or insulin resistance (measured as HOMA) as phenotype. Alternatively, BMI, leptin concentration or metabolic rate have been used as quantitative traits. In obese Mexican Americans, linkage was observed between fat mass or leptin concentrations and a region on chromosome 2 (Comuzzie *et al* 1997). POMC is the most interesting candidate gene in this chromosomal region. In the Quebec family study, linkage was found between percentage body fat and a region on the long arm of chromosome 20. This region is syngenic to a region on mouse chromosome 2, to which linkage was first observed (Lembertas *et al* 1997). More recently, linkage between basal metabolic rate and a region on chromosome 11 was reported in the Quebec study; this region includes both ucp2 and ucp3 genes.

6.6 THE PRESENT STUDY

From the preceding chapters it is clear that obesity is a worldwide problem and is a factor in the pathogenesis of the MS and kidney disease. Certainly the development of obesity-related hypertension and other neuro-hormonal mechanisms that include the action of leptin and other inflammatory mediators, undoubtedly play important roles in this pathogenesis.

Although several studies have investigated the association or relationship of the LEP gene with some obesity phenotypes (commonly BMI) and to the serum concentrations of leptin, only one study has studied this gene and its association with a specific disease viz. the cardiovascular disease atherosclerosis, which was defined by the thickness of the carotid wall (carotid intima medial thickness (CIMT)). However, there appear to be no focussed studies that have looked at the association of the LEP gene with kidney disease phenotypes despite the evidence (see chapter 5) that the gene product may be involved in the pathogenesis of kidney disease.

Moreover, there are no reports in literature of the study of this gene in the black African population. Several reports of study on this gene have been in Caucasians and in a few cases in African Americans. Considering the fact that black Africans have a disproportionately higher prevalence of kidney disease than people of other races (see Chapter 4), and because obesity is becoming very common in Africa, especially in urbanised Africans we designed a study to analyse the effects of obesity associated with the MS on kidney disease. The study was also designed to define the relationship between the LEP gene and kidney disease in the black African population of South Africa.

AIM:

Due to the afore mentioned relationship between leptin and kidney disease (chapter 5), the aim of this thesis is in establishing whether an association exists between the LEP gene and kidney disease phenotypes (independent of diabetes and hypertension) in this homogenous black African population.

OBJECTIVES:

The objectives of this thesis therefore include:

1. To assess the effect of the MS on kidney disease phenotypes in this population
2. Identifying the prevalence of genotypes of the LEP in black South Africans.
3. To identify the relationship between the LEP gene, obesity, hypertension, the MS, markers of kidney disease and serum leptin concentration in black Africans.
4. To identify candidate genes for the MS through a bioinformatics approach.

The next three chapters form the bulk of my experimental work and address these objectives as follows:

1. An investigation and analysis of the phenotypes of the MS, obesity, and kidney disease and their correlations in a black (South) African population (chapter 7).
2. An analysis of the LEP gene resulting in establishing the genotype–phenotype associations between different SNPs and MS, obesity, and kidney disease phenotypes (chapter 8).
3. A computational – bioinformatics assessment through "mining" various databases in the public domain in order to find other candidate genes for MS genes (chapter 9).

CHAPTER 7

THE METABOLIC SYNDROME AND KIDNEY DISEASE

7.1 INTRODUCTION:

The MS is strongly associated with the risk for diabetes, CVD including coronary artery disease and stroke and with the risk for all-cause mortality. The relationship between insulin resistance and kidney disease has been amply reported, however, there are not many studies that have reported on the relationship between the MS and kidney disease. Hoehner and colleagues (2002) in a cross-sectional survey of 934 non-diabetic Native Americans found that the insulin resistance syndrome was associated with microalbuminuria and that persons with 3 or more traits for the insulin resistance syndrome had 2.3-fold higher odds of microalbuminuria than persons with no traits (Hoehner *et al* 2002).

Chen *et al* (2004) were the first to report on the relationship between the MS and kidney disease in a large sample of Americans (Chen *et al* 2004). Using data of the NHANES III, they identified a strong, positive, and significant relationship between the MS and risk for CKD and microalbuminuria. Although the study of Chen *et al* (2004) did not reveal the fraction of their cohort that had diabetes, (diabetes alone being a potent risk for CKD and microalbuminuria), they were however able to show a significant relationship between the MS and CKD after excluding all diabetics (Chen *et al* 2004).

The public health impact of CKD / ESRD has been examined. ESRD requiring RRT comes at great cost to the patient and the society and reports have shown that blacks suffer more from hypertension related-ESRD, although the reasons for this are still unclear. The Medical Research Council of South Africa conducted a survey in 2000 and reported that CVDs

(including nephritis and nephroses) are the second cause of death (16.6%), second only to HIV/AIDS which accounted for 30% of total deaths. The number of years of life lost from NCDs amounted to 21% (Bradshaw *et al* 2003).

The MS presents an opportunity for looking at other factors (aside form hypertension alone) that may potentially steer the higher prevalence of kidney disease in people of the black race. A recent Pubmed search on the relationship between the MS and kidney disease in any native black African population has yielded no reports or studies in this field.

We thus set about to investigate the relationship between phenotypes of the MS and kidney disease in a native black (South) African population.

7.2 METHODOLOGY

7.2.1. ETHICS

The study was approved by the research ethics committee of the University of Cape Town in 2005 (REC REF: 043/2005).

7.2.2. SAMPLE SIZE ESTIMATION

A pilot study was carried out in the hypertension clinic of the Groote Schuur Hospital (GSH) with the approval of Prof Brian Rayner, head of the clinic. This was done to enable us have an estimate of the number of subjects that will be required for the full study. Data was collected from the case notes of about 40 black Xhosa subjects attending this clinic. The data included age, gender, blood pressures, height and weight and laboratory variables including plasma creatinine, glucose (fasting), lipids and urine ACR. Using this data, it was estimated that 269 subjects will be needed to provide the study with sufficient power (80%) to obtain statistically significant results which are unlikely to have occurred by chance.

7.2.3. STUDY LOCATION AND POPULATION

South Africa with its rich array of ethnic backgrounds is made up of about 79% Black Africans, 9.6% Whites, 8.9% Coloureds and 2.5% Indians/Asians. The black population of South Africa is also quite diverse. The Xhosas (relevant to this study) are the second largest of the black population (17.6%) after the Zulus who make up 23.8% of the blacks of South Africa (Byrnes 1996).

The homeland of the Xhosas is the Eastern Cape (formerly Transkei and Ciskei) and due to its proximity to the Western Cape, the majority of black South Africans in the townships of the Western Cape are Xhosas who had migrated to this city in search of a job or had been born here (Byrnes 1996).

In the Western Cape, several informal settlements or townships had been set up to cater for the needs of those coming in search of a job. Gugulethu is one of such townships and is one of the oldest black townships in Cape Town and South Africa.

Established in 1958 as Nyanga West as a result of the migrant labour system of the former South African government, Gugulethu grew as the number of migrant workers from the Transkei increased and Langa (a nearby black township) became too small to accommodate them all. Many of the people in this township still have their families in the Eastern Cape (Transkei area), a reflection of the past in which hostel dwellers were not allowed to bring family members.

Gugulethu has a community health centre which operates a daily medical out patients' clinic, a 24 hour trauma unit, a HIV/AIDS treatment centre and a twice weekly hypertension and diabetes clinic with frequent referrals to the GSH.

This study was conducted at the Gugulethu hypertension clinic between May 2005 and July 2006 and was of a cross-sectional design. Consecutive patients attending this clinic were informed of the nature of this study and those willing to participate were screened for diabetes by blood glucose testing after a 12 hour fast and after signing the consent forms of the study (see appendix (A3) for consent forms).

Through this process, 273 hypertensive subjects were screened with 84 of them requiring the oral glucose tolerance test (OGTT) to exclude diabetes. Eight of the subjects were excluded on account of incident diabetes mellitus after the OGTT; 12 others declined to continue the study for various reasons. Overall, 253 hypertensive subjects were recruited for the study. The OGTT involved the oral administration of 75g of glucose in 300ml of water to subjects with impaired fasting glucose defined as fasting glucose $\geq$ 5.6 mmol/L. Blood glucose was drawn again from such subjects at time 60 minutes and 120 minutes. Subjects with 2 hour blood glucose $\geq$ 11.1mmol/L were classified as diabetics and excluded from the study. Such subjects were then referred to the diabetes clinic for further assessment and treatment.

As the overall aim of this thesis is to test whether an association exists between the LEP gene and kidney disease phenotypes (independent of diabetes and hypertension), non-diabetic and normotensive first degree relatives of the hypertensives were also invited to take part in the study and were therefore included in the analysis of the relationship between MS phenotypes and kidney disease markers. Eighty-three (83) first degree relatives were included in the

study after screening for diabetes and hypertension. Eventually, 336 subjects were recruited in total, comprising 253 non-diabetic probands (hypertensives) and 83 (non-diabetic) first degree normotensive relatives. However, only 334 subjects were used in the analysis as results could not be obtained for 2 of the subjects who were also unavailable for a repeat of the tests.

A questionnaire was administered to all participants (see appendix A4) in order to obtain information on age (date of birth), sex, ethnicity, smoking, alcohol and physical activity, duration of hypertension, history of CVD and family history of hypertension. Height, weight, waist and hip circumference were measured and recorded (as described in the next section below).

Subjects were not screened for HIV, despite the high prevalence of HIV amongst black South Africans and the obvious complication of HIV-associated nephropathy (HIVAN) that could result from it. This was because it would be considered unethical screening for HIV for the purpose of this study, especially as the theme is not HIV/AIDS related. However, as subjects with HIVAN often present with symptoms of uraemia, heavy proteinuria, severe oedema, and other symptoms of immunodeficiency, such subjects were essentially excluded as subjects with infections or chronic diseases requiring treatment were routinely excluded. Furthermore, subjects on highly active anti-retroviral therapy (HAART) were specifically excluded from the study to ensure that all cases of lipodystrophy arising from the use of HAART were excluded to avoid confounding factors.

Pregnant women were also excluded from participating as pregnancy is associated with an altered haemodynamic state with increased fluid retention, renal blood flow and GFR and could be a potential confounding factor.

Patients with known secondary causes of hypertension (commonly hyperaldosteronism or presence of a mutation in the epithelial sodium channel [R563Q] first described by Prof Brian Rayner in the Cape population) (Rayner et al. 2003) were also excluded from the study as were all those with malignant hypertension.

Finally, although any black population would have been targeted for this study, the choice of the Xhosas was to ensure a homogenous population sharing similar alleles inherited from a common ancestor, i.e. a population that was identical-by-decent (IBD) rather than one in which the disease gene is expected to occur by chance (Lander and Schork, 1994). Ethnicity was determined by the individual's home language, which is the language of his/her parents and not just any other acquired language and we confirmed this in the questionnaire. Hence, all black subjects of other nationalities or black South Africans who were not Xhosa were excluded from the study.

7.2.4. CLINICAL ASSESSMENT OF SUBJECTS:

7.2.4.1 Waist and Hip Circumferences:

To measure WC, a measuring tape was placed snugly in a horizontal plane around the abdomen at the level of the iliac crest. The measuring tape was parallel to the floor and the measurement was taken at the end of a normal expiration to the nearest 0.1cm. Hip circumference was measured at the level of the greater trochanters. This position is generally taken as the widest circumference below the WC. It was also measured using the tape measure and was reported to the nearest 0.1 cm. The WHR is the ratio of both

circumferences. All measurements were done by the same person, using the same tape measure throughout the period of the study.

7.2.4.2 Body Mass Index

The BMI was calculated as weight (kg) divided by height squared (m^2)

7.2.4.3 Blood Pressure

This was taken using a validated Accouson mercury sphygmomanometer. Patients were made to sit comfortably for a period of no less than five minutes. Blood pressure was then taken in both arms and the arm with the greater reading was used for assessment. Two blood pressure measurements were taken from this arm two minutes apart and the average of the two readings was taken as the blood pressure reading. Mean arterial blood pressure (MAP) was calculated from these readings using the equation:

MAP = DBP + Pulse pressure / 3 (mmHg)

Pulse pressure was calculated from SBP – DBP (mmHg) (Ganong 1981)

7.2.5. LABORATORY ASSESSMENT:

Blood was drawn by a trained research nurse and sent to the National Health Laboratory Service (NHLS) who handled all the biochemical and hormonal analysis. Spot urine was also taken for measuring the urine ACR.

Routine chemistry included:

Lipid profile: Cholesterol, Triglycerides (TG), High density lipoprotein cholesterol (HDL), Low density lipoprotein cholesterol (LDL)

Liver function: Albumin

Renal function: Creatinine, urine microalbumin, urine creatinine.

Endocrine functions: Glucose, Insulin, Leptin, Adiponectin,

Inflammatory and prothrombotic markers: Fibrinogen, C-reactive protein (CRP).

For all these tests, conventional assays were used in the NHLS chemical pathology laboratory on auto-analysers with appropriate quality control.

7.2.5.1 Glomerular Filtration Rate (GFR)

This was calculated using the Modification of Diet in Renal Disease (MDRD) equation:

$GFR = 186 \times (P_{Cr})^{-1.154} \times (age)^{-0.203} \times (0.742 \text{ if female}) \times (1.210 \text{ if black})$

Although the MDRD Study equation has not been validated in subjects <18years, pregnant women, the elderly (age >70 years), racial or ethnic subgroups other than Caucasians and African Americans, in individuals with normal kidney function who are at increased risk for CKD, or in normal individuals it was still the preferred method for calculating the GFR. This is because GFR estimates using this equation are more accurate than serum creatinine alone; moreover, the MDRD Study equation has been rigorously developed and validated and it is more accurate than measured creatinine clearance from 24-hour urine collections (Levey *et al*. 2000). Table 7.1 shows estimates of GFR for stratification of age groups in decades.

Table 7.1: Estimates of GFR for stratified age groups (Levey *et al* 2000).

Age (years)	Average estimated GFR (ml/min)
20-29	116
30-39	107
40-49	99
50-59	93
60-69	85
70+	75

7.2.5.2 Homeostasis Model Assessment (HOMA):

Although the most appropriate method for assessing insulin resistance is the euglycaemic clamp method. HOMA has been shown to correlate with the degree of insulin resistance from different populations.

It is calculated from the following equation:

Fasting glucose * Fasting Insulin / 22.5

The beta-cell function (%B) and the insulin sensitivity (%S) were estimated using the Diabetes Trial Unit (The Oxford centre for Diabetes, Endocrinology and Metabolism) online model assessment calculator (www.dtu.ox.ac.uk).

7.2.5.3 Kidney Biopsy

Although it had been intended that kidney biopsy would be performed in subjects with massive proteinuria, no such subject was identified during the course of this study. Kidney biopsy might illustrate if there are unique histological features peculiar to the MS and might have demonstrated if the features were similar or the same as features seen in obesity related glomerulopathy.

7.2.5.4 Definition of the Metabolic Syndrome

The MS was defined using cutoffs of the ATPIII as already discussed in chapter 3 of this thesis (see Table 3.2).

7.2.6 STATISTICAL ANALYSIS

The data was first put into Microsoft Excel spreadsheets and exported into SPSS statistical software (SPSS version 11.0 Chicago USA). Continuous data were analysed using the students t-test. Analysis of variance (ANOVA) was used to compare the means of multiple variables. Categorical data were analysed in contingency tables using χ^2. Individual methods of analysis are indicated in the texts. The results were expressed as percentages or as mean ± SEM except if otherwise stated. In some cases where data was skew, logarithmic (log) transformation was done to adjust the data appropriately, however in such cases, the real values and not the log transformed values were used. Correlation coefficients (partial and regression) which in general statistical usage refers to the departure of two variables from independence were used to indicate the strength and direction of linear relationship between variables. Although a p value of <0.05 is arbitrarily accepted worldwide as significant (unlikely to have occurred by chance), it is the conventional value for statistical significance and is used in this thesis as the cutoff for statistical significance.

7.3 RESULTS

The demographic features of the study participants are as shown in table 7.2. Male subjects made up 34.1% of the entire study population and the mean age of participants significantly and proportionally rose with increasing number of MS components. Due to differences in age, sex, and duration of hypertension, the p value was adjusted for these factors and also given in the tables.

Table 7.2: Demographic features of the study participants.

VARIABLE	NUMBER OF MS TRAITS					p	p§
	0	1	2	3	≥4		
N=334 (%)	36 (10.8)	56 (16.8)	130 (38.9)	73 (21.9)	39 (11.6)		
Age (yrs)	31.7 (2.3)	46.3 (2.0)	53.7 (1.0)	55.2 (1.2)	55.9 (1.7)	<0.0001	_
Males (%)	18 (50.0)	34 (60.7)	32 (24.6)	24 (32.9)	6 (15.4)	<0.0001	_
Yrs of Education (yrs)	8.9 (0.5)	6.9 (0.5)	6.2 (0.2)	6.3 (0.3)	5.6 (0.5)	<0.0001	0.009
Physical Activity (Hours/week)	4.5 (0.2)	4.5 (0.2)	4.4 (0.1)	4.2 (0.2)	4.2 (0.2	0.708	0.763
Current Smokers (%)	4.2	6.0	4.5	3.9	0.9	<0.0001	0.149
Alcohol users (%)	5.1	8.4	9.3	6.3	2.1	0.002	0.700
Duration of Hypertension (yrs)	0	2.1 (0.3)	3.4 (0.1)	3.4 (0.2)	3.7 (0.2)	<0.0001	_

N – Number of subjects

Values indicate mean (SEM).

p values obtained from ANOVA of mean values.

p§ - p value adjusted for differences in age, sex , duration of hypertension and use of anti-hypertensive medications.

All the blood pressure measurements (SBP, DBP, MAP and Pulse pressure) significantly increased as components of the MS increased. However, after adjusting the p value for age, sex and differences in duration of hypertension, pulse pressure was no longer significantly different between the groups although it increased with increasing number of MS traits (Table 7.3)

Table 7.3: Clinical features of the study participants.

VARIABLE	NUMBER OF MS TRAITS					p	p§
	0	1	2	3	≥4		
(N=334) (%)	36 (10.8)	56 (16.8)	130 (38.9)	73 (21.9)	39 (11.6)		
SBP (mmHg)	124.5 (2.4)	138.4(3.3)	148.2(1.8)	153.2(2.2)	161.1(1.7)	<0.0001	0.001
DBP(mmHg)	81.7 (1.5)	86.8(1.6)	93.4(1.0)	95.0(1.4)	100.5(6.8)	<0.0001	<0.0001
MAP (mmHg)	95.9 (1.7)	104.0 (2.1)	111.6 (1.2)	112.8(2.2)	117.4 (2.0)	<0.0001	<0.0001
Pulse Pressure (mmHg)	42.9 (1.9)	51.6 (2.3)	54.9 (1.3)	57.5 (1.8)	57.1 (2.4)	<0.0001	0.319
BMI (kg/m^2)	23.9 (0.9)	27.4 (0.8)	35.4 (0.8)	36.3 (0.8)	37.7 (1.1)	<0.0001	<0.0001
WC (cm)	80.9 (1.5)	92.8 (2.0)	109 (1.3)	114.2(1.5)	115.8(2.0)	<0.0001	<0.0001
Hip (cm)	97.8 (1.8)	105.3(2.0)	120.9(1.6)	122.2(1.8)	127.8(2.4)	<0.0001	<0.0001
WHR	0.83 (0.06)	0.88 (0.09)	0.91 (0.08)	0.94(0.08)	0.91 (0.06)	<0.0001	<0.0001

N – Number of subjects

Values indicate mean (SEM).

p values obtained from ANOVA of mean values.

p§ - p value adjusted for differences in age, sex, duration of hypertension and use of anti-hypertensive medications.

SBP – Systolic blood pressure, DBP – Diastolic blood pressure, MAP – Mean arterial pressure, BMI – Body mass index, WC – Waist circumference, Hip – Hip circumference, WHR – Waist to hip ratio.

Table 7.4.1 to 7.4.3 shows the biochemical features of all the study participants. Fasting glucose was significantly higher in subjects with the MS (3 or more traits). The 1 hour and 2 hour post glucose load measurements were not different between the groups. Insulin, %S, and HOMA all increased with increasing MS traits and were significantly different at first, but again after adjusting the p value for age, sex and duration of hypertension were no longer different between the groups.

All measured serum lipids including TG, HDL, LDL and Cholesterol remained significantly higher (HDL lower) as the number of MS traits increased. (Table 7.4.2)

Table 7.4.1: Biochemical features of the study participants

VARIABLE	NUMBER OF MS TRAITS					P	P§
	0	1	2	3	≥4		
(N=334) (%)	36 (10.8)	56 (16.8)	130 (38.9)	73 (21.9)	39 (11.6)		
FBG (mmol/L)	4.7 (0.1)	4.7 (0.1)	5.0 (0.1)	5.5 (0.1)	5.8 (0.1)	<0.0001	<0.0001
60 Minutes***	7.6	8.1 (1.2)	9.5 (0.5)	9.0 (0.5)	9.6 (0.5)	0.646	0.427
120 Minutes***	5.5	6.2 (0.5)	7.9 (0.4)	7.7 (0.4)	7.9 (0.3)	0.207	0.874
Insulin (mU/L)	7.0 (0.7)	7.3 (1.0)	15.2 (1.8)	26.5 (4.6)	48.0 (11.1)	<0.0001	0.857
%B	113.8 (8.5)	112.3 (11.1)	121.2 (4.6)	134.2(8.9)	140.9(11.9)	0.153	0.752
%S	123.7 (10.6)	134.2 (10.2)	97.3 (5.0)	76.5 (6.9)	58.3 (6.1)	<0.0001	0.773
HOMA	1.5 (0.1)	1.6 (0.2)	3.9 (0.7)	7.2 (1.4)	14.4 (3.5)	<0.0001	0.746
Albumin (g/L)	46.0 (0.4)	44.7 (0.4)	43.9 (0.3)	44.1 (0.3)	43.7 (0.5)	0.005	0.072

N – Number of subjects

*** The number of subjects who had OGTT and participated was 65.

Values indicate mean (SEM).

p values obtained from ANOVA of mean values.

P§ - p value adjusted for differences in age, sex , duration of hypertension and use of anti-hypertensive medications.

FBG – Fasting blood glucose, %B – Estimated beta cell function, %S – Estimated insulin sensitivity, HOMA – Homeostasis model assessment

Table 7.4.2: Biochemical features of the study participants

VARIABLE	NUMBER OF MS TRAITS					P	P§
	0	1	2	3	≥4		
(N=334) (%)	36 (10.8)	56 (16.8)	130 (38.9)	73 (21.9)	39 (11.6)		
TG (mmol/L)	0.7 (0.1)	1.0 (0.1)	1.2 (0.1)	1.6 (0.1)	2.1 (0.1)	<0.0001	<0.0001
HDL (mmol/L)	1.7 (0.1)	1.7 (0.1)	1.5 (0.1)	1.3 (0.1)	1.2 (0.1)	<0.0001	<0.0001
LDL (mmol/L)	2.0 (0.1)	2.4 (0.1)	2.9 (0.1)	2.9 (0.1)	3.2 (0.2)	<0.0001	<0.0001
Total Cholesterol (mmol/L)	4.0 (0.1)	4.5 (0.1)	4.9 (0.1)	4.9 (0.1)	5.3 (0.2)	<0.0001	0.003

N – Number of subjects

Values indicate mean (SEM).

p values obtained from ANOVA of mean values.

P§ - p value adjusted for differences in age, sex , duration of hypertension and use of anti-hypertensive medications.

TG – Triglycerides, HDL – High density lipoprotein cholesterol, LDL – Low density lipoprotein cholesterol

Age and sex adjusted level of CRP was not significantly different between the different groups stratified according to number of MS traits. However, fibrinogen, a prothrombotic factor and marker for systemic inflammation was significantly higher in subjects with the MS. (Table 7.4.3)

Both leptin and adiponectin [with the leptin –adiponectin ratio (LAR)] significantly increased with increasing number of MS traits. This remained true for adiponectin and LAR after age and sex adjustment for the p-value. However, this significance was lost for leptin after this adjustment which could indicate that one of these factors (age, sex, hypertension duration) was responsible for its higher level in subjects with more MS components (Table 7.4.3).

Table 7.4.3: Biochemical features of the study participants

VARIABLE	NUMBER OF MS TRAITS					P	P§
	0	1	2	3	≥4		
(N=334) (%)	36 (10.8)	56 (16.8)	130 (38.9)	73 (21.9)	39 (11.6)		
CRP (mg/L)	4.3 (1.3)	8.0 (2.3)	9.7 (0.9)	10.7 (1.3)	9.1 (1.3)	0.109	0.684
Fibrinogen (g/L)	3.4 (0.1)	3.7 (0.1)	4.2 (0.1)	4.2 (0.1)	4.3 (0.1)	<0.0001	0.033
Adiponectin (μg/ml)	8.7 (1.0)	9.1 (0.7)	8.0 (0.4)	5.4 (0.5)	5.8 (0.7)	<0.0001	<0.0001
Leptin (ng/ml)	14.3 (2.5)	18.7 (2.6)	35.8 (2.3)	34.9 (2.8)	36.9 (3.4)	<0.0001	0.198
LAR	2.1 (0.4)	3.5 (0.7)	6.1 (0.5)	9.7 (1.3)	10.0 (1.6)	<0.0001	<0.0001

N – Number of subjects

Values indicate mean (SEM).

p values obtained from ANOVA of mean values.

P§ - p value adjusted for differences in age, sex , duration of hypertension and use of anti-hypertensive medications.

CRP – C-reactive protein, LAR – Leptin to Adiponectin ratio.

Serum creatinine tended to rise proportionally with MS traits but this was not significantly different between the different groups. Mean GFR difference was approximately 30% (133.9 ml/min/1.73m^2 to 94.4 ml/min/1.73m^2) between subjects who had no MS trait to those with 4 or more traits and this difference remained significant after adjusting for age, sex and duration of hypertension (p=0.028) (Table 7.5).

Albumin-to-creatinine ratio increased four-fold from subjects with no MS traits to those with four or more traits and the incidence of microalbuminuria was higher in subjects with the MS. Four subjects had overt proteinuria. Two of these were in the group with 2 MS traits (1.5%) and the other 2 in the group with 4 MS traits (5.1%) (Table 7.5).

Table 7.5: Markers of renal functions in the study participants

VARIABLE	NUMBER OF MS TRAITS					P	P§
	0	1	2	3	≥4		
(N=334) (%)	36 (10.8)	56 (16.8)	130 (38.9)	73 (21.9)	39 (11.6)		
Creatinine (µmol/L)	71.7 (3.0)	80.2 (3.8)	84.4 (4.7)	75.8 (2.2)	90.0 (11.8)	0.256	0.773
GFR (ml/min/1.73m^2)	133.9 (6.1)	112.9 (3.5)	100.6 (2.6)	103.9(2.6)	94.4 (3.0)	<0.0001	0.028
ACR (mg/mmol)	1.4 (0.5)	1.2 (0.2)	2.8 (0.6)	3.3 (0.6)	4.7 (1.8)	<0.0001	0.021
Microalbuminuria (%)	11.4	12.3	18.9	28.2	23.7	0.019	0.087
Clinical proteinuria (%)	_	_	1.5	_	5.1	_	_

N – Number of subjects , Values indicate mean (SEM).

p values obtained from ANOVA of mean values.

P§ - p value adjusted for differences in age, sex , duration of hypertension and use of anti-hypertensive medications.

GFR- glomerular filtration rate, ACR- albumin-to-creatinine ratio.

A student's t-test was used to compare the clinical and biochemical features of hypertensive and normotensive subjects according to the presence (ACR^+) or absence (ACR^-) of microalbuminuria. The total incidence of microalbuminuria was 19.5% and as would be expected, hypertensive subjects had a greater incidence of microalbuminuria compared with normortensive subjects. Blood pressure (SBP and DBP), fasting glucose and urine ACR were significantly higher in microalbuminuric hypertensives than in normoalbuminuric hypertensives ($p<0.05$ in all cases) (Table 7.6). Microalbuminuric normotensives had significantly elevated values of urine ACR compared with normoalbuminuric normotensive subjects (Table 7.6).

TABLE 7.6: Characteristics of subjects by presence or absence of microalbuminuria

	HYPERTENSIVES		NORMOTENSIVES	
	ACR$^+$	**ACR$^-$**	**ACR$^+$**	**ACR$^-$**
N (%)	54 (21.5)***	197 (78.5)	11 (13.3)***	72 (86.7)
Age (yrs)	55.4 (1.3)	56.2 (0.7)	37.4 (4.0)	34.2 (1.5)
SBP (mmHg)	159.2 (2.7)	150.5 (1.4)**	127.3 (5.3)	126.9 (2.0)
DBP (mmHg)	98.5 (1.6)	93.9 (0.8)**	83.6 (2.1)	82.9 (1.3)
WC (cm)	110.0 (2.5)	108.7 (1.1)	97.0 (7.4)	93.2 (2.1)
Fasting Glucose (mmol/L)	5.4 (0.1)	5.2 (0.1)**	4.9 (0.2)	4.7 (0.1)
TG (mmol/L)	1.5 (0.1)	1.4 (0.1)	1.3 (0.2)	0.9 (0.1)
HDL (mmol/L)	1.5 (0.1)	1.4 (0.03)	1.5 (0.2)	1.7 (0.1)
Leptin (ng/ml)	33.0 (3.5)	31.8 (1.7)	28.9 (7.7)	24.9 (2.5)
Creatinine (µmol/L)	92.4 (10.8)	79.6 (1.5)	72.1 (4.3)	71.1 (3.0)
GFR (ml/min)	100.4 (4.2)	99.1 (1.6)	113.6 (8.2)	129.7 (4.1)
ACR (mg/mmol)	10.9 (1.5)	0.8 (0.1)**	9.6 (2.3)	0.7 (0.1)*

N – Number of subjects
Values indicate mean (SEM).
ACR$^+$ Indicates subjects with microalbuminuria

ACR$^-$ Indicates subjects without microalbuminuria.

* Indicates significant difference between normoalbuminuric and microalbuminuric normotensive subjects

** Indicates significant difference between normoalbuminuric and microalbuminuric hypertensive subjects.

*** Indicates significant difference between microalbuminuric hypertensive and normotensive subjects

SBP- systolic blood pressure, DBP- diastolic blood pressure, BMI- body mass index, WC- waist circumference, HOMA – Homeostasis model assessment, TG- triglycerides, HDL- high density lipoprotein, CRP- C-reactive protein, GFR- glomerular filtration rate, ACR- albumin-to-creatinine ratio, ACR$^+$ is presence of microalbuminuria, ACR$^-$ is normoalbuminuria.

A partial correlation matrix was used to analyse the relationship between MS and both urine ACR (Table 7.7.1) and estimated GFR (Table 7.7.2). MS phenotypes were used as the variables, and differences in age, sex, and differences in duration of hypertension were controlled for.

In subjects with the MS, SBP, DBP and Fasting glucose positively and significantly correlated with ACR whereas an inverse but significant correlation was observed between GFR with BMI and serum Leptin. In subjects without the MS, DBP and fasting TG modestly and positively correlated with urine ACR.

Table 7.7.1: Partial correlation coefficients of urine ACR with metabolic variables in MS^+ and MS^- subjects.

	Urine ACR	
	MS^+	**MS^-**
SBP (mmHg)	0.255*	0.126
DBP (mmHg)	0.206*	0.143*
BMI (kg/m^2)	0.078	- 0.088
WC (cm)	- 0.013	- 0.037
TG (mmol/L)	0.062	0.156*
HDL (mmol/L)	0.115	0.008
Fasting glucose (mmol/L)	0.230*	0.084
Leptin (ng/ml)	- 0.076	-0.023

MS^+ Indicates subjects with the MS.

MS^- Indcates subjects without the MS

* Indicates significant correlation between variable and ACR

SBP- systolic blood pressure, DBP- diastolic blood pressure, BMI- body mass index, WC- waist circumference, TG- triglycerides, HDL- high density lipoprotein, ACR- albumin-to-creatinine ratio.

Table 7.7.2: Partial correlation coefficients of estimated GFR with metabolic variables in MS^+ and MS^- subjects.

	Estimated GFR	
	MS^+	**MS^-**
SBP (mmHg)	0.059	- 0.031
DBP (mmHg)	- 0.023	- 0.057
BMI (kg/m^2)	- 0.223**	0.098
WC (cm)	- 0.131	- 0.017
TG (mmol/L)	0.007	- 0.111
HDL (mmol/L)	- 0.001	0.031
Fasting glucose (mmol/L)	- 0.073	- 0.111
Leptin (ng/ml)	- 0.272**	- 0.091

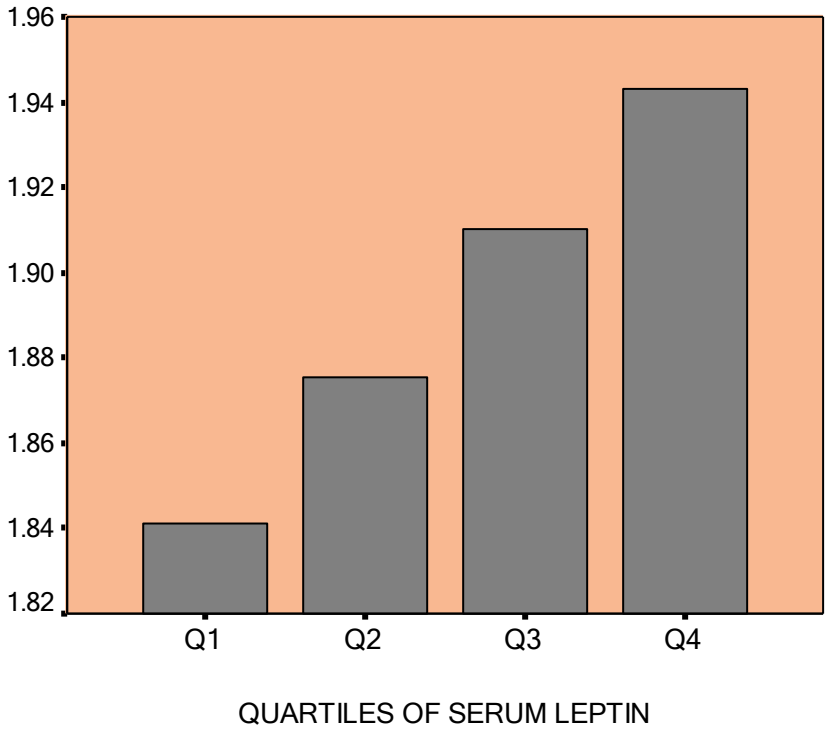
MS^+ Indicates subjects with the MS.

MS^- Indcates subjects without the MS

** Indicates significant correlation between variable and GFR

SBP- systolic blood pressure, DBP- diastolic blood pressure, BMI- body mass index, WC- waist circumference, TG- triglycerides, HDL- high density lipoprotein, GFR- glomerular filtration rate.

Figure 7.1: Bar chart depicting the relationship between serum leptin (in quartiles) and Log transformed ACR after adjusting for confounding variables.



The relationship between serum leptin (quartiles) and microalbuminuria measured as urine ACR (log transformed) is represented in the bar chart in. ACR is seen to linearly increase across the four quartiles of serum leptin concentrations.

Logistic regression analysis was used to determine the odds ratio (OR) for microalbuminuria with individual and several components of the MS. High SBP (≥130 mmHg), high DBP (≥ 85 mmHg) and high fasting serum TG (≥1.7mmol/L) had significant OR of 2.59 (95%CI of 1.18-5.70); 3.33 (95% CI of 1.52-7.29) and 1.99 (95% CI of 1.08-3.66) respectively. The OR

for microalbuminuria increased with number of MS traits and was significant in subjects with the MS (Table 7.8).

Table 7.8: Logistic regression analysis for predictors of microalbuminuria using individual and several MS components.

PREDICTOR	OR	95% CI	p
Large WC	1.22	0.66, 2.25	0.518
High SBP	2.59	1.18, 5.70	0.015
High DBP	3.33	1.52, 7.29	0.002
IFG	1.78	0.86, 3.71	0.119
High TG	1.99	1.08, 3.66	0.026
Low HDL-C	1.06	0.57, 1.99	0.851
1 Component	0.53	0.23, 1.23	0.136
2 Components	0.95	0.55, 1.66	0.863
3 Components	1.91	1.04, 3.51	0.035
4 Components	1.21	0.53, 2.80	0.651
5 Components	4.20	0.36, 64.10	0.272
Metabolic syndrome	1.89	1.09, 3.30	0.023

7.4 DISCUSSION

In the Strong Heart Study of non-diabetic Native Americans, the MS did not predict an increase in incident CVD events (Resnick *et al*., 2003). However, results from other large population-based studies that included diabetics have shown that the presence of the MS is associated with a significantly increased risk of all-cause mortality and CVD morbidity and mortality (Lakka *et al* 2002). This study, the first of its kind in a non-diabetic and homogenous native black African population has some important renal observations that include: (1) A four-fold rise in ACR from subjects with no MS traits to those with 4 or more traits (table 7.5), (2) a significant decline in GFR with increasing number of MS traits (Table 7.5), (3) an increasing odds ratio (likelihood) for the occurrence of the microalbuminuria with increasing MS phenotypes (Table 7.8), (4) ACR and GFR in MS subjects correlating significantly with SBP, DBP, fasting glucose; and with BMI and leptin respectively (Table 7.7.1 and 7.7.2) (5) High SBP, high DBP and hypertriglyceridaemia being the independent predictors of microalbuminuria (Table 7.8).

Microalbuminuria implies endothelial dysfunction and may herald impending and serious renal damage. In hypertensives microalbuminuria is a marker of target organ damage whereas in diabetics it generally marks the beginning of nephropathy. In this population, the finding of an almost four fold rise in ACR from subjects with no MS traits to those with 4 or more traits clearly shows that additional metabolic risk factors, even in the absence of diabetes, increase the risk for kidney disease. This increase in ACR may mark the initiation of kidney damage, seen as significant decline in GFR with increasing MS traits.

Also, the positive and significant correlation of ACR with SBP and DBP in subjects with the MS and DBP in subjects without the MS highlights the importance of blood pressure in the

aetiology of kidney disease in black Africans. Although blood pressure alone is a significant cause of kidney disease, especially in black Africans, it might need the presence of other risk factors to accelerate the onset and progression of kidney disease. This concept was tested by Mule *et al* (2005) who studied the effects of additional risk factors on target organ damage caused by hypertension in a group of white hypertensives. They found that the occurrence of the MS may amplify hypertension related cardiac and renal damage over and above that which may result from individual contributions from each component.

The studies of Salako *et al* (2007) in Nigeria and Rayner and Becker (2006) in South Africa have shown that microalbuminuria is common in treated and untreated hypertensive black Africans and both studies pointed out the implications of adequate BP control with regards to kidney disease. However, although kidney disease arising from hypertension tends to be common amongst people of African origin, hypertension alone may not account for the higher prevalence of kidney disease seen in blacks.

Therefore, the significant inverse correlation observed for GFR with BMI and serum leptin (Table 7.7.2) and the linear increase of urine ACR across the four quartiles of serum leptin (Figure 7.1) may implicate obesity as a factor for renal disease in this population. Chen *et al*. (2004) have shown that the risk for being affected by CKD was more than twice as high in patients with an increased WC as in those without, suggesting that obesity may be an independent risk factor for CKD. Although the exact mechanisms that link obesity and renal damage have not yet been understood fully, it is suggested that some of the hormones secreted by fat cells (e.g. leptin, IL-6 and TNF-α) may be partly involved in promoting renal injury (Wisse 2004). Leptin, for instance, is strongly correlated with the degree of obesity and

has been shown to predispose to glomerulosclerosis (Wolf *et al* 1999) and increased urine albumin excretion in women with type 1 diabetes (Rudberg and Persson 1998).

This association suggests that obesity, both phenotypically and through the actions of its hormones, may play a significant role in initiating and leading to the progression of kidney disease in black Africans. The observation may also strongly support the Kincaid-Smith hypothesis (2004) that argued that renal disease in black Africans may be linked more closely to obesity and insulin resistance than to blood pressure alone as is generally supposed.

Therefore, as hypertension alone may not explain or account for the majority of kidney disease cases seen worldwide, especially in black Africans who are more likely to be labelled as having hypertension-related ESRD, it has become necessary to explore other factors, (environmental or genetic) that may contribute to the increased incidence and progression of kidney disease amongst blacks. The MS as a "single" entity provides an opportunity of finding such environmental factors and has been shown in this study to be associated with kidney disease. A potential genetic factor that may account for the higher prevalence of hypertension related kidney disease in black Africans could be the LEP gene (see chapter 5 for effects of leptin on blood pressure and the kidney). This genetic influence is sought in the next chapter.

CHAPTER 8

ASSOCIATION STUDY OF THE LEP GENE AND VARIOUS PHENOTYPES THAT IMPACT ON CARDIOVASCULAR DISEASE RISK.

8.1 INTRODUCTION

The increasing prevalence of obesity seen in many populations of the world and its major complications, especially CVD which is the principal source of morbidity and mortality in obese people, prompted a search for candidate genes that play a role in the pathophysiology of CKD and obesity.

As discussed in chapter 6, several reports with conflicting results have been published on the association between the human LEP gene located in chromosome 7 and obesity phenotypes and serum leptin concentration. Despite the conflicting results, the leptin-signalling pathway is regarded as one of the most important routes in energy homeostasis in human metabolism (Tritos and Mantzoros 1997).

There are very few studies that have sought for the existence of associations between polymorphisms of the LEP gene and CVD markers (other than obesity) given the identified role of obesity in CVDs generally. Also, there are no reports of studies in native black Africans on the relationship between the LEP gene and any marker of CVD.

Three polymorphisms in the 5' untranslated region (5' UTR) of the LEP gene (G2548A, C188A, and A19G) have been studied and reported often from different populations. G2548A and C188A are in the promoter region of the gene and A19G in the non-coding exon 1. Also, a rare polymorphism in the non-coding sequence of exon 3, the C538T, has been reported only twice in the literature and was reported by Gaukrodger *et al* (2005) to be associated with

a lower incidence of atherosclerosis in Caucasians. These four polymorphisms, having been previously studied in other populations were chosen, of the approximately 140 SNPs in the LEP gene, for the present study. Although these polymorphisms are in the non-coding regions of the LEP gene, studies on similar positioned polymorphisms from other genes have shown that they may influence the expression of the gene and its protein (Misquitta *et al* 2001, Komar 2007).

These polymorphisms were thus chosen for the current study, firstly because there have been reports about them from other populations providing a basis for comparison with results from our study. Secondly, leptin, the obesity gene product has been shown to play a role in obesity and hypertension and to cross-talk with insulin pathways and therefore could play a role in insulin resistance and the MS (see chapter 5). Thirdly, chronic leptin elevation has been shown to increase renal sympathetic nerve activity and to play a role in glomerulosclerosis and proteinuria (see chapter 5). Even though hyperleptinaemia has been shown to be related with renal disease, it is not known if the same association with renal disease exists with the LEP gene.

Thus, in this part of the study we attempted an analysis of the LEP gene in order to establish the genotype–phenotype relationships between the selected SNPs and MS, obesity, hypertension and particularly kidney disease phenotypes.

8.2 METHODOLOGY:

The recruitment process, sample size estimation, study population, and assessment of clinical and laboratory variables have been described in chapter 7. It is imperative to state here that the sample size for this part of the study was not separately estimated but was based on sample size obtained from the part described in chapter 7. Also, sample sizes for the different SNPs examined differed and were fewer than that of the entire study population and depended on availability of high quality DNA, successfully completed genotyping experiments or (in)ability to get the subject again for a repeat examination. Thus, there is variation in sample sizes of the different SNPs. Sample numbers analysed were: 250 – (G2548A [rs7799039]); 293 - (C188A [rs791620]); 293 - (A19G [rs2167270]) and 300 – (C538T [ENSSNP5824596]). For the purpose of simplicity, these genotypes will not be described in the text by their RefSeq ids. Consent was obtained from the subjects to have their blood genetically tested.

8.2.1 GENETIC ANALYSIS:

All the genetic analysis was carried out at the Human genetics Laboratory (Ramesar Lab) of the University of Cape Town. This involved DNA extraction, primer design, polymerase chain reaction (PCR) for DNA amplification and restriction enzyme (RE) digests of the amplified DNA.

8.2.2 DNA EXTRACTION:

Genomic DNA was isolated from peripheral blood lymphocytes using the Puregene DNA Isolation Kit (Gentra Systems, USA) according to the manufacturer's protocol.

Specifically, the white cells from peripheral blood are separated from the red cells and lysed with a strong detergent to release their DNA content. Cellular proteins also released along

with the DNA from the white cells, and are removed with the enzyme proteinase K to prevent their interfering with downstream molecular experiments. The extraction process is completed by washing the DNA with ethanol and finally eluting with water. DNA purity and yield was confirmed using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies Inc Welmington USA)

8.2.3 POLYMERASE CHAIN REACTION AND RESTRICTION ENZYME DIGESTS

Polymerase Chain Reaction (based on the enzymatic amplification of a DNA fragment that is flanked by two stretches of nucleotide primers, which hybridise to opposite strands of the sequence being investigated) was carried out individually for the 4 SNPs being tested. This was followed by RE digest on obtained PCR products. The REs are enzymes isolated from bacteria that recognize specific sequences of DNA and then cut the DNA to produce fragments called restriction fragments. The method employed for the PCR amplification across the 4 SNPs being analysed, along with the protocol for RE digests are as shown in the appendices A1.1 to A1.4.

Briefly, amplicons of sizes 109 base pairs (bp) (G2548A), 261bp (C188A), 258bp (A19G) and 316bp (C538T) were generated by PCR. For G2548A, the RE HhaI which cuts the amplicon at position 62 was used to generate 2 fragments (62bp and 47bp - genotype GG) or 3 bands (109bp, 62bp and 47bp - genotype GA) or one band if the amplicon is undigested (109bp - genotype AA). Similarly, the amplicon of the C188A polymorphism was digested with AscI which cuts at position 221 producing 2 fragments (221bp and 40bp - genotype CC) or 3 bands (261bp, 221bp and 40bp – genotype CA) while the undigested amplicon would be seen as one band (261bp – genotype AA). Also, the A19G polymorphism which had an amplicon size of 258bp was digested with Mspa1I cutting it at position 186 and generating 2 bands (186bp and 62bp – genotype GG) or 3 bands (258bp, 186bp and 62bp – genotype AG)

or in the case of the undigested amplicon, one band (258bp – genotype AA). The RE TaiI cut the C538T amplicon at 2 positions (32 and 245) thus producing 3 bands (213bp, 76bp and 32bp – genotype CC) for the completely digested PCR products. Partial digests gave rise to 4 bands (289bp, 213bp, 76bp and 32bp – genotype CT) while undigested amplicon was seen as 2 bands (289bp and 32bp).

8.2.4 STATISTICS

Analysis of variance (ANOVA) was used to compare the means of multiple variables. Categorical data were analysed in contingency tables using χ^2. Three models were used in the contingency tables to determine the χ^2 values and level of significance (p values) between subjects grouped according to the presence or absence of hypertension, obesity, the MS, microalbuminuria, difference in gender and high or normal serum leptin level. The first model assumes equal dominance of the genotypes of the SNP being studied (co-dominant model), the second (dominant model) assumes the homozygous genotype of the ancestral allele as dominant and compares this genotype with the sum of the heterozygous genotype and the homozygous recessive genotype. The third model (recessive model) compares the recessive genotype with the sum of the heterozygous and the homozygous dominant/ancestral genotypes. These models could not be applied to the G2548A and C538T polymorphisms in which only 2 genotypes were obtained. The results were expressed as percentages or as mean (SEM) except where otherwise stated. Logistic regression was used to determine the estimate of the odds ratio at 95% confidence interval of the means.

R and R packages genetics (version 1.3.8.) and haplo.stats were used to perform haplotype analyses. R is a free software environment for statistical computing and graphics. R is available from http://www.r-project.org. Linkage disequilibrium (LD) between each pair of the G2548A, C188A, A19G and C538T SNPs was assessed by calculating the disequilibrium statistics D, *D', r,* Chi-squared test statistic and the p-value for the test of no LD (the sign of

D’ is always positive, D and r have negative signs when the minor allele in one SNP is associated with the major allele in the other). Haplo.stats is a set of functions for the analysis of indirectly measured haplotypes. Specifically, the method described by Sinnwell *et al* (2008) was used. The statistical methods assume that all subjects are unrelated and that haplotypes are ambiguous (due to unknown linkage phase of the genetic markers). The functions in haplo.stats are used for the estimation of haplotype frequencies, posterior probabilities of haplotype pairs for a subject, GLM regression models for the regression of a trait on haplotypes, model relationships between haplotypes and a wide variety of traits, including binary, ordinal, quantitative, and Poisson. The coefficient (β) in the model summary is the effect of that specific haplotype compared to the base (most common or assumed wild type) haplotype. The effect is the estimated difference in the mean of the transformed phenotype between that haplotype and the reference haplotype. A significant p-value (< 0.05) means that it has a significant difference with the base haplotype.

8.3 RESULTS

Genotype frequencies at all polymorphisms satisfied Hardy-Weinberg equilibrium (used to relate the frequencies of genotypes at a single mendelian locus to the phenotype frequencies in a population) and agreed closely with genotype frequencies described at these polymorphisms in black Africans in the Hapmap project (The International HapMap Consortium 2003) (Table 8.1(a)). Table 8.1(b) provides genotype and allele comparison with Hapmap data from black Africans of the Yoruba (YRI) ethnic group in West Africa. Minor but non-significant differences in allele and genotype frequencies can only be seen in the C188A polymorphism suggesting that black Africans may have similar occurrence of these polymorphisms.

Table 8.1(a): Genotype and allele frequencies of the 4 SNPs in black South Africans.

	Genotype Frequency – N (%)			**Allele Frequency**	
	GG	**GA**	**AA**	**G**	**A**
G2548A [rs7799039]	221 (88.4)	29 (11.6)	0 (0)	0.942	0.058
	CC	**CA**	**AA**	**C**	**A**
C188A [rs791620]	248 (84.6)	40 (13.7)	5 (1.7)	0.871	0.129
	GG	**AG**	**AA**	**G**	**A**
A19G [rs2167270]	78 (26.6)	166 (56.7)	49 (16.7)	0.549	0.451
	CC	**CT**	**TT**	**C**	**T**
C538T*	261 (86.7)	39 (13.0)	0 (0)	0.935	0.065

N – Number of subjects

Ancestral alleles – G2548A – G, C188A – C, A19G – G, C538T – C.

* Currently there is no RefSeq id for this polymorphism

Table 8.1(b) Genotype Frequencies of black population (Yoruba - YRI) from the Hapmap project.

SNP	Genotypes (Frequency)			Alleles (Frequency)	
G2548A	AA (0)	AG (0.034)	GG (0.966)	A (0.017)	G (0.983)
C188A	AA (0)	AC (0)	CC (1.000)	A (0)	C (1.000)
A19G	AA (0.136)	AG (0.591)	GG (0.273)	A (0.475)	G (0.525)
C538T*	CC	CT	TT	C	T

* There are currently no Hapmap genotype frequencies available for this SNP and no RefSeq id.

8.3.1 ANALYSIS OF THE LEP A19G GENOTYPES WITH A VARIETY OF STUDIED PHENOTYPES.

Tables 8.2.1 to 8.2.3 show, respectively, the co-dominant, dominant and recessive models in comparing the frequencies of the 3 genotypes obtained for the polymorphism at exon 1 of the LEP gene (A19G). There was no difference in patterns of the genotype distribution between subjects categorised by differences in blood pressure (hypertensive / normotensive), obesity, MS (present or absent), kidney disease marker (microalbuminuric / normoalbuminuric), gender, and serum leptin concentration (high / low) in all three models.

Although as shown in table 8.3, analysis of variance (ANOVA) of the mean values of the clinical and biochemical parameters of subjects in the 3 genotype groups did not reach statistical significance (at a p value of <0.05), we did observe a consistent pattern in which subjects who were homozygous for the A allele almost always had higher mean values of the

clinical and biochemical variables compared to the homozygous GG or the heterozygous group.

Furthermore, studying the association between these genotypes and markers of kidney disease [serum creatinine concentration, estimated GFR, and urine albumin-to-creatinine (ACR) concentration], it was observed that GFR declined steadily from the homozygous dominant genotype (GG) to the homozygous recessive genotype (AA) and ACR increased in the same trend, with the recessive genotype having a modest but significantly higher mean ACR concentration (p=0.04) (Table 8.4.1a).

In the probands, the homozygote genotype "AA" had a higher GFR compared to subjects with the homozygote "GG" or heterozygotes "AG", however, this was not statistically significant (p = 0.658). Those with genotype "AA" also had higher urine ACRcompared to the other two groups, but this only approached significance (p = 0.078) (Table 8.4.1b).

This trend was sought for in the offspring (total of 50) of all the probands that had participated in the study and whose LEP A19G genotype had been ascertained (Table 8.4.2). Again, the estimated GFR was lowest in the homozygous recessive group (AA) and the urine ACR was significantly higher in this same group (p = 0.026).

Table 8.2.1: Analysis of the LEP A19G Genotypes with variables of interest using the co-dominant model.

	GG % (N)	**AG % (N)**	**AA % (N)**	χ^2	**P**
HYPERTENSIVES	26.9 (59)	54.8 (120)	18.3 (40)	1.80	0.408
NORMOTENSIVES	25.7 (19)	62.2 (46)	12.2 (9)		
OBESE	26.7 (48)	53.4 (93)	16.7 (16)	1.91	0.385
NON-OBESE	25.6 (30)	60.7 (71)	13.7 (16)		
MS PRESENT	25.0 (24)	58.3 (56)	16.7 (16)	0.19	0.912
MS ABSENT	27.2 (53)	55.9 (109)	16.9 (33)		
ACR^+	19.2 (10)	57.7 (30)	23.1 (12)	2.73	0.256
ACR^-	27.9 (67)	56.7 (136)	15.4 (37)		
MALES	27.6 (29)	60.0 (63)	12.5 (13)	2.23	0.328
FEMALES	26.1 (49)	54.8 (103)	19.1 (36)		
HIGH LEPTIN	27.3 (57)	54.5 (114)	18.2 (38)	1.82	0.402
NORMAL LEPTIN	22.8 (18)	63.3 (50)	13.9 (11)		

N – Number of subjects

MS – Metabolic syndrome; ACR^+ Microalbuminuria; ACR^- Normoalbuminuria

Table 8.2.2: Analysis of the LEP A19G Genotypes with variables of interest using the dominant model.

	GG % (N)	**AG + AA % (N)**	χ^2	**P**
HYPERTENSIVES	26.9 (59)	73.1 (160)	0.05	0.831
NORMOTENSIVES	25.7 (19)	74.3 (55)		
OBESE	26.7 (48)	72.4 (126)	0.14	0.713
NON-OBESE	25.6 (30)	74.4 (87)		
MS PRESENT	25.0 (24)	75.0 (72)	0.16	0.692
MS ABSENT	27.2 (53)	72.8 (142)		
ACR^+	19.2 (10)	80.8 (42)	1.66	0.131
ACR^-	27.9 (67)	72.1 (173)		
MALES	27.6 (29)	72.4 (76)	0.08	0.438
FEMALES	26.1 (49)	73.9 (139)		
HIGH LEPTIN	27.3 (57)	72.7 (152)	0.60	0.439
NORMAL LEPTIN	22.8 (18)	77.2 (61)		

N – Number of subjects

MS – Metabolic syndrome; ACR^+ Microalbuminuria; ACR^- Normoalbuminuria

Table 8.2.3: Analysis of the LEP A19G Genotypes with variables of interest using the recessive model.

	AA % (N)	**GG + AG % (N)**	χ^2	**P**
HYPERTENSIVES	18.3 (40)	81.7 (179)	1.48	0.150
NORMOTENSIVES	12.2 (9)	87.8 (65)		
OBESE	16.7 (16)	81.0 (141)	1.40	0.237
NON-OBESE	13.7 (16)	86.3 (101)		
MS PRESENT	16.7 (16)	83.2 (80)	0.003	0.956
MS ABSENT	16.9 (33)	83.1 (162)		
ACR^+	23.1 (12)	76.9 (40)	1.80	0.180
ACR^-	15.4 (37)	86.6 (203)		
MALES	12.5 (13)	87.6 (92)	2.22	0.137
FEMALES	19.1 (36)	80.9 (152)		
HIGH LEPTIN	18.2 (38)	81.8 (171)	0.74	0.391
NORMAL LEPTIN	13.9 (11)	86.1 (68)		

N – Number of subjects

MS – Metabolic syndrome; ACR^+ Microalbuminuria; ACR^- Normoalbuminuria

Table 8.3: Analysis of the LEP A19G genotypes with clinical and biochemical features of all the subjects.

	GG (N=78)	AG (N=166)	AA (N=49)	P
Age (years)	50.3 (1.8)	51.0 (1.1)	49.8 (1.8)	0.844
BMI (kg/m^2)	33.4 (1.0)	32.6 (0.7)	34.0 (1.3)	0.526
WC (cm)	104.8 (2.0)	104.5 (1.3)	105.2 (2.3)	0.973
WHR	0.90 (0.01)	0.90 (0.01)	0.90 (0.01)	0.904
SBP (mmHg)	144.5 (2.6)	145.2 (1.7)	151.3 (3.2)	0.192
DBP (mmHg)	91.9 (1.4)	91.6 (0.9)	94.9 (1.7)	0.224
FBG (mmol/L)	5.0 (0.1)	5.2 (0.1)	5.2 (0.1)	0.370
Insulin (mU/L)	18.3 (2.5)	17.4 (2.6)	28.4 (7.9)	0.160
HOMA	4.6 (0.70	4.8 (0.9)	7.9 (2.4)	0.196
TG (mmol/L)	1.3 (0.1)	1.4 (0.1)	1.1 (0.1)	0.207
HDL-C (mmol/L)	1.5 (0.1)	1.5 (0.03)	1.6 (0.1)	0.184
CRP (mg/L)	9.5 (1.4)	9.5 (1.0)	7.5 (1.1)	0.567
Fibrinogen (g/L)	4.1 (0.1)	4.0 (0.1)	4.1 (0.1)	0.272
Leptin (ng/ml)	32.0 (2.9)	28.4 (1.9)	32.8 (3.5)	0.392
Adiponectin (μg/ml)	7.7 (0.6)	7.3 (0.4)	6.7 (0.6)	0.516

N – Number of subjects

Values are presented as mean (SEM).

SBP- systolic blood pressure, DBP- diastolic blood pressure, BMI- body mass index, WC- waist circumference, HOMA – Homeostasis model assessment, TG- triglycerides, HDL- high density lipoprotein, CRP- C-reactive protein, GFR- glomerular filtration rate, ACR- albumin-to-creatinine ratio, ACR$^+$ is presence of microalbuminuria, ACR$^-$ is normoalbuminuria.

Table 8.4.1a: Analysis of the LEP A19G genotypes with markers of renal function in all the subjects.

	GG (N=78)	AG (N=166)	AA (N=49)	P
CREATININE (µmol/L)	78.6 (3.0)	82.0 (3.8)	75.0 (2.4)	0.525
GFR (ml/min/1.73m^2)	106.6 (3.5)	105.5 (2.3)	104.9 (3.3)	0.941
ACR (mg/mmol)	1.4 (0.3)	2.4 (0.4)	3.7 (1.1)	**0.049**

N – Number of subjects

Values are given as mean (SEM)

Table 8.4.1b: Analysis of the LEP A19G genotypes with markers of renal function in the probands.

	GG (N=58)	AG (N=120)	AA (N=40)	P
GFR (ml/min/1.73m^2)	99.9 (2.8)	97.8 (2.3)	101.9 (3.5)	0.658
ACR (mg/mmol)	1.3 (0.3)	2.8 (0.5)	3.6 (1.2)	0.078

N – Number of subjects

Values are given as mean (SEM)

Table 8.4.2: Analysis of the LEP A19G genotypes with markers of renal function in normotensive offspring of the probands.

	GG (N=17)	AG (N=27)	AA (N=6)	P
CREATININE (µmol/L)	75.2 (10.9)	66.8 (2.9)	72.5 (4.8)	0.626
GFR (ml/min/1.73m^2)	134.2 (11.4)	138.1 (5.5)	119.6 (5.6)	0.526
ACR (mg/mmol)	2.0 (0.9)	0.8 (0.3)	5.7 (4.0)	**0.026**

N – Number of subjects

Values are given as mean (SEM)

Table 8.5: Univariate Logistic Odds Ratios of obesity, the metabolic syndrome and microalbuminuria associated with LEP A19G polymorphism

SNP / GENOTYPES		BMI	MS	ACR
A19G	GG	1.11 (0.65 – 1.88)	0.89 (0.51 – 1.56)	0.62 (0.29 – 1.30)
	AG	0.74 (0.46 – 1.18)	1.12 (0.68 – 1.83)	1.00 (0.55 – 1.82)
	AA	1.47 (0.77 – 2.81)	0.99 (0.51 – 1.90)	1.61 (0.60 – 4.35)

Values are given as Estimate (95%CI)

Univariate logistic regression odds ratio (OR) was calculated as an estimate of the relative risk for obesity, the MS and microalbuminuria associated with the LEP A19G genotypes. In keeping with the earlier observation in which subjects with the recessive genotype (AA) had significantly elevated urine ACR, they also had the highest risk for microalbuminuria OR=1.61 (95% CI - 0.60 to 4.35) compared with the dominant [OR=0.62 (95% CI – 0.29 - 1.30)] and heterozygous [OR=1.00 (95% CI – 0.55 – 1.82)] genotypes. The relative risk for obesity was highest for subjects with the AA genotype: OR=1.47 (95% CI – 0.77 – 2.81) (Table 8.5).

8.3 .2 ANALYSIS OF THE LEP GENE PROMOTER REGION (G2548A AND C188A) GENOTYPES WITH A VARIETY OF STUDIED PHENOTYPES.

For both polymorphisms in the LEP gene promoter region, there was no difference in the frequency of occurrence of the genotypes (GG/GA and CC/CA/AA) in the participants when they were categorised by blood pressure, obesity, MS, microalbuminuria, gender, and serum leptin concentration (Tables 8.6 and 8.7.1 to 8.7.3). Although this lack of difference was still present when the C188A genotypes were analysed as co-dominant, dominant and recessive models, the dominant model (CC vs. CA + AA) showed a trend towards significance with regards to obesity (p = 0.084) suggesting a possible relationship between the dominant genotype and obesity.

Table 8.8 shows the univariate logistic regression odds ratio of obesity, the MS and microalbuminuria associated with the genotypes of the C188A polymorphism. This table shows that the OR for obesity is significantly higher in subjects of the dominant genotype i.e. CC: (OR=1.88, 95% CI – 1.03 to 3.46; p = 0.039)

All the clinical and biochemical features, including obesity phenotypes, blood pressure, blood glucose, insulin and assayed cytokines (leptin and adiponectin) did not differ between the genotypes of the G2548A and C188A polymorphisms (Tables 8.9 and 8.10).

Table 8.6: Analysis of the LEP G2548A Genotypes with variables of interest.

	GG % (N)	GA % (N)	χ^2	P
HYPERTENSIVES	87.4 (167)	12.6 (24)	0.74	0.391
NORMOTENSIVES	91.5 (54)	8.5 (5)		
OBESE	85.4 (129)	14.6 (22)	3.09	0.079
NON-OBESE	92.8 (90)	7.2 (7)		
MS PRESENT	86.4 (76)	13.6 (12)	0.50	0.480
MS ABSENT	89.4 (143)	10.6 (17)		
ACR^+	86.7 (39)	13.3 (6)	0.15	0.697
ACR^-	88.7 (181)	11.3 (23)		
MALES	86.3 (82)	13.7 (13)	0.65	0.420
FEMALES	89.7 (181)	10.3 (16)		
HIGH LEPTIN	89.4 (160)	10.6 (19)	0.44	0.510
NORMAL LEPTIN	86.4 (57)	13.6 (9)		

N – Number of subjects

MS – Metabolic syndrome; ACR^+ Microalbuminuria; ACR^- Normoalbuminuria

Table 8.7.1: Analysis of the LEP C188A Genotypes with variables of interest using the Co-dominant model.

	CC % (N)	CA % (N)	AA % (N)	χ^2	P
HYPERTENSIVES	84.2 (187)	13.5 (30)	2.3 (5)	1.63	0.443
NORMOTENSIVES	85.9 (61)	14.1 (10)	0 (0)		
OBESE	87.5 (154)	10.8 (19)	1.7 (3)	3.28	0.194
NON-OBESE	80.0 (92)	18.3 (21)	1.7 (2)		
MS PRESENT	84.7 (83)	13.3 (13)	2.0 (2)	0.12	0.944
MS ABSENT	84.5 (163)	14.0 (27)	1.6 (3)		
ACR^+	87.5 (49)	12.5 (7)	0 (0)	1.28	0.528
ACR^-	84.3 (199)	13.2 (32)	2.1 (5)		
MALES	80.0 (84)	17.1 (18)	2.9 (3)	3.15	0.207
FEMALES	87.2 (164)	11.7 (22)	1.1 (2)		
HIGH LEPTIN	86.3 (182)	12.3 (26)	1.4 (3)	1.04	0.596
NORMAL LEPTIN	81.8 (63)	15.6 (12)	2.6 (2)		

N – Number of subjects

MS – Metabolic syndrome; ACR^+ Microalbuminuria; ACR^- Normoalbuminuria

Table 8.7.2: Analysis of the LEP C188A Genotypes with variables of interest using the dominant model.

	CC % (N)	**CA + AA % (N)**	**χ^2**	**P**
HYPERTENSIVES	84.2 (187)	15.8 (35)	0.12	0.732
NORMOTENSIVES	85.9 (61)	14.1 (10)		
OBESE	87.5 (154)	12.5 (22)	2.99	0.084
NON-OBESE	80.0 (92)	20.0 (23)		
MS PRESENT	84.7 (83)	15.3 (15)	0.003	0.958
MS ABSENT	84.5 (163)	15.6 (30)		
ACR^+	87.5 (49)	12.5 (7)	0.36	0.550
ACR^-	84.3 (199)	15.3 (37)		
MALES	80.0 (84)	20.0 (21)	2.71	0.100
FEMALES	87.2 (164)	12.8 (24)		
HIGH LEPTIN	86.3 (182)	13.7 (29)	0.88	0.350
NORMAL LEPTIN	81.8 (63)	18.2 (14)		

N – Number of subjects

MS – Metabolic syndrome; ACR^+ Microalbuminuria; ACR^- Normoalbuminuria

Table 8.7.3: Analysis of the LEP C188A Genotypes with variables of interest using the Recessive model.

	AA % (N)	**CA + CC % (N)**	χ^2	**P**
HYPERTENSIVES	2.3 (5)	97.7 (217)	1.63	0.202
NORMOTENSIVES	0 (0)	100.0 (71)		
OBESE	1.7 (3)	98.3 (173)	0.001	0.984
NON-OBESE	1.7 (2)	98.3 (113)		
MS PRESENT	2.0 (2)	98.0 (96)	0.09	0.763
MS ABSENT	1.6 (3)	97.9 (190)		
ACR^+	0 (0)	100.0 (56)	1.21	0.272
ACR^-	2.1 (5)	97.9 (231)		
MALES	2.9 (3)	97.1 (102)	1.29	0.256
FEMALES	1.1 (2)	98.9 (186)		
HIGH LEPTIN	1.4 (3)	98.6 (208)	0.46	0.499
NORMAL LEPTIN	2.6 (2)	97.4 (75)		

N – Number of subjects

MS – Metabolic syndrome; ACR^+ Microalbuminuria; ACR^- Normoalbuminuria

Table 8.8: Univariate Logistic Odds Ratios of obesity, the metabolic syndrome and microalbuminuria associated with LEP C188A polymorphism

SNP / GENOTYPES		BMI	MS	ACR
C188A	CC	1.88 (1.03 – 3.46)	1.22 (0.63 – 2.36)	1.51 (0.64 – 3.57)
	CA	0.56 (0.29 – 1.09)	0.97 (0.48 – 1.98)	0.94 (0.39 – 2.25)
	AA	1.00 (0.17 – 6.09)	1.36 (0.22 – 8.28)	0.81 (0.77 – 0.86)

Values are given as Estimate (95% CI)

Table 8.9: Analysis of the LEP G2548A with clinical and biochemical features of all the subjects.

	GG (N=221)	GA (N=29)	P
Age (years)	52.0 (0.9)	49.6 (2.7)	0.378
BMI (kg/m^2)	33.3 (0.6)	34.5 (1.6)	0.488
WC (cm)	105.5 (1.2)	106.9 (3.3)	0.679
WHR	0.90 (0.01)	0.92 (0.02)	0.346
SBP (mmHg)	146.3 (1.5)	150.9 (5.2)	0.394
DBP (mmHg)	92.4 (0.8)	95.3 (2.8)	0.319
FBG (mmol/L)	5.1 (0.1)	5.3 (0.2)	0.332
Insulin (mU/L)	18.3 (2.3)	14.8 (2.8)	0.580
HOMA	5.0 (0.8)	3.7 (0.8)	0.542
TG (mmol/L)	1.3 (0.1)	1.3 (0.1)	0.615
HDL-C (mmol/L)	1.5 (0.03)	1.4 (0.09)	0.270
CRP (mg/L)	10.0 (0.9)	9.7 (2.0)	0.916
Fibrinogen (g/L)	4.1 (0.1)	4.0 (0.2)	0.622
Leptin (ng/ml)	30.9 (1.7)	27.6 (4.6)	0.505
Adiponectin (µg/ml)	7.4 (0.3)	6.6 (1.2)	0.453
Creatinine (µmol/L)	81.4 (3.0)	80.9 (4.3)	0.960
GFR (ml/min/1.73m^2)	104.2 (1.9)	106.0 (6.2)	0.754
ACR (mg/mmol)	2.7 (0.4)	2.0 (0.5)	0.573

N – Number of subjects

Values are presented as mean (SEM)

SBP- systolic blood pressure, DBP- diastolic blood pressure, BMI- body mass index, WC- waist circumference, HOMA – Homeostasis model assessment, TG- triglycerides, HDL- high density lipoprotein, CRP- C-reactive protein, GFR- glomerular filtration rate, ACR- albumin-to-creatinine ratio, ACR$^+$ is presence of microalbuminuria, ACR$^-$ is normoalbuminuria.

Table 8.10: Analysis of LEP C188A Genotypes with clinical and biochemical features of all the subjects.

	CC (N=248)	CA (N=40)	AA (N=5)	P
Age (years)	51.0 (0.9)	51.1 (2.4)	58.8 (2.5)	0.463
BMI (kg/m^2)	33.4 (0.6)	32.0 (1.5)	32.3 (3.5)	0.606
WC (cm)	105.5 (1.1)	102.5 (3.3)	103.0 (0.5)	0.572
WHR	0.91 (0.01)	0.89 (0.01)	0.92 (0.03)	0.460
SBP (mmHg)	146.5 (1.4)	143.0 (3.8)	163.8 (9.1)	0.142
DBP (mmHg)	92.5 (0.8)	91.4 (1.9)	101.0 (3.1)	0.250
FBG (mmol/L)	5.2 (0.1)	5.1 (0.1)	4.9 (0.3)	0.608
Insulin (mU/L)	20.3 (2.4)	14.5 (2.9)	13.1 (6.9)	0.585
HOMA	5.6 (0.8)	3.6 (0.8)	3.0 (0.9)	0.541
TG (mmol/L)	1.3 (0.1)	1.4 (0.1)	2.0 (0.7)	0.082
HDL-C (mmol/L)	1.5 (0.03)	1.4 (0.1)	1.2 (0.2)	0.280
CRP (mg/L)	9.5 (0.7)	9.3 (2.5)	2.8 (0.8)	0.475
Fibrinogen (g/L)	4.1 (0.1)	3.9 (0.2)	3.6 (0.2)	0.350
Leptin (ng/ml)	30.8 (1.5)	28.0 (4.3)	20.7 (7.1)	0.543
Adiponectin (µg/ml)	7.3 (0.3)	7.7 (0.7)	5.2 (0.8)	0.619
Creatinine (µmol/L)	80.4 (2.7)	78.4 (3.4)	88.4 (7.4)	0.862
GFR (ml/min/1.73m^2)	105.2 (1.9)	108.0 (5.0)	89.4 (3.0)	0.398
ACR (mg/mmol)	2.6 (0.4)	2.6 (0.8)	0.4 (0.1)	0.663

N – Number of subjects

Values are presented as mean (SEM)

SBP- systolic blood pressure, DBP- diastolic blood pressure, BMI- body mass index, WC- waist circumference, HOMA – Homeostasis model assessment, TG- triglycerides, HDL- high density lipoprotein, CRP- C-reactive protein, GFR- glomerular filtration rate, ACR- albumin-to-creatinine ratio, ACR^+ is presence of microalbuminuria, ACR^- is normoalbuminuria.

8.3.3 ANALYSIS OF LEP C538T GENOTYPES WITH A VARIETY OF STUDIED PHENOTYPES.

As only two genotypes (CC / CT) were obtained for the polymorphism in exon 3, only the co-dominant model was used in the analysis of the genotype frequencies. No difference was observed in the frequency of the genotypes when the subjects were grouped according to blood pressure, obesity, MS, microalbuminuria, gender, and serum leptin concentration (high or low) (Table 8.11).

Furthermore, comparison of the mean values of the clinical and biochemical features revealed no difference in age, obesity phenotypes (BMI, WC, WHR), blood pressures (SBP and DBP), lipids, inflammatory markers and assayed cytokines (Table 8.12). Subjects with the T allele i.e. subjects with genotype CT appeared to have "better" mean values of serum creatinine, estimated GFR, and with a significantly lower mean urine ACR ($p = 0.001$).

In table 8.13, the subjects were divided into two groups: proband and offspring and comparison of the markers of kidney function was carried out between the genotypes for these two groups. In the probands, GFR was significantly higher (better) in the CT group (108.7 ± 4.0 vs 97.1 ± 1.8; $p = 0.010$) and ACR was also significantly lower in the CT group (1.4 ± 0.3 vs 3.0 ± 0.5; $p < 0.004$). This was also the observed trend in the offspring although the values did not reach significance.

The odds ratio for microalbuminuria in subjects with the T allele (CT genotype) was 0.62 (95% CI – 0.23 to 1.66), three-fold lower than in subjects with the dominant genotype CC (Table 8.14).

Table 8.11: Analysis of the LEP C538T Genotypes with variables of interest.

	CC % (N)	**CT % (N)**	χ^2	**P**
HYPERTENSIVES	86.2 (193)	13.8 (31)	0.55	0.458
NORMOTENSIVES	88.3 (68)	10.4 (8)		
OBESE	85.4 (152)	14.6 (26)	0.90	0.344
NON-OBESE	88.4 (107)	10.7 (13)		
MS PRESENT	82.8 (82)	17.2 (17)	2.17	0.140
MS ABSENT	88.5 (177)	11.0 (22)		
ACR^+	89.3 (50)	8.9 (5)	0.93	0.335
ACR^-	86.1 (210)	13.9 (34)		
MALES	85.2 (92)	13.9 (15)	0.15	0.696
FEMALES	87.6 (169)	12.4 (24)		
HIGH LEPTIN	87.5 (189)	12.5 (27)	0.37	0.546
NORMAL LEPTIN	83.8 (67)	15.0 (12)		

N – Number of subjects

MS – Metabolic syndrome; ACR^+ Microalbuminuria; ACR^- Normoalbuminuria

Table 8.12: Analysis of LEP C538T Genotypes with clinical and biochemical features of all the subjects.

	CC (N=261)	**CT (N=39)**	**P**
Age (years)	50.6 (0.9)	52.4 (2.1)	0.455
BMI (kg/m^2)	33.1 (0.5)	33.6 (1.4)	0.721
WC (cm)	104.9 (1.1)	105.1 (2.6)	0.930
WHR	0.90 (0.01)	0.90 (0.01)	0.611
SBP (mmHg)	145.8 (1.4)	147.4 (3.7)	0.670
DBP (mmHg)	91.9 (0.8)	93.6 (1.8)	0.436
FBG (mmol/L)	5.1 (0.1)	5.3 (0.1)	0.078
Insulin (mU/L)	18.2 (1.9)	28.0 (9.7)	0.329
HOMA	4.9 (0.6)	7.7 (2.8)	0.353
TG (mmol/L)	1.3 (0.1)	1.3 (0.1)	0.964
HDL-C (mmol/L)	1.5 (0.03)	1.5 (0.1)	0.806
CRP (mg/L)	9.0 (0.7)	10.1 (2.0)	0.605
Fibrinogen (g/L)	4.0 (0.1)	4.1 (0.2)	0.795
Leptin (ng/ml)	30.7 (1.6)	27.0 (3.1)	0.381
Adiponectin (µg/ml)	7.6 (0.3)	6.2 (0.7)	0.060
Creatinine (µmol/L)	81.3 (2.6)	72.5 (2.6)	0.195
GFR (ml/min/1.73m^2)	104.8 (1.9)	110.5 (3.5)	0.258
ACR (mg/mmol)	2.7 (0.4)	1.3 (0.2)	**0.001**

N – Number of subjects

Values presented as mean (SEM)

SBP- systolic blood pressure, DBP- diastolic blood pressure, BMI- body mass index, WC- waist circumference, HOMA – Homeostasis model assessment, TG- triglycerides, HDL- high density lipoprotein, CRP- C-reactive protein, GFR- glomerular filtration rate, ACR- albumin-to-creatinine ratio, ACR$^+$ is presence of microalbuminuria, ACR$^-$ is normoalbuminuria.

Table 8.13: Analysis of LEP C538T Genotypes with markers of renal function in probands and their offspring.

	PROBANDS		OFFSPRING	
	CC (N=193)	**CT (N=31)**	**CC (N=49)**	**CT (N=5)**
CREATININE (µmol/L)	84.8 (3.3)	72.1 (3.0)	70.2 (4.1)	66.8 (4.5)
GFR (ml/min/1.73m^2)	97.1 (1.8)	108.7 (4.0)*	135.3 (4.9)	130.0 (7.1)
ACR (mg/mmol)	3.0 (0.5)	1.4 (0.3)**	1.8 (0.6)	0.9 (0.5)

N – Number of subjects
Values given as mean (SEM), GFR – Glomerular filtration rate, ACR – Albumin to creatinine ratio.
* P = 0.010
** P = 0.004

Table 8.14: Univariate Logistic Odds Ratios of obesity, the metabolic syndrome and microalbuminuria associated with LEP C538T polymorphism

SNP / GENOTYPES		BMI	MS	ACR
C538T	**CC**	0.71 (0.35 – 1.45)	0.60 (0.30 – 1.19)	1.62 (0.60 – 4.35)
	CT	1.41 (0.69 – 2.84)	1.67 (0.84 – 3.31)	0.62 (0.23 – 1.66)

Values presented as Estimate (95%CI)

8.3.4: Haplotype Analysis

There are 16 different possible haplotypes that can be constructed from 4 bi-allelic markers. Only 6 were observed in this sample, their inferred haplotype frequencies are shown in the bar chart in figure 8.1. A haplotype "case-control" comparison was done (cases = probands, controls = relatives). The observed differences between the haplotype frequencies of cases and controls were not significant (Table 8.15.). The GCGC haplotype had the highest frequency of occurrence in the probands (42%) while the haplotype GCAC occurred more frequently in the relatives (46%).

There was a significant difference between the GCAT and the base haplotype (GCGC) in markers of renal function in the probands. Table 8.16a shows the summaries of linear regression of haplotypes on phenotypes of interest in probands. The GCAT haplotype decreases plasma creatinine ($\beta = -0.41$; $p = 0.0279$) and increases GFR ($\beta = 0.46$; $p = 0.0173$) compared to the GCGC haplotype. Although the GCAT decreases urine ACR, this effect was not significant in the probands ($\beta = -0.12$; $p = 0.5960$). Also, this haplotype (GCAT) had no significant effect on BMI ($\beta = 0.35$; $p = 0.0514$) or serum leptin concentration ($\beta = 0.07$; $p = 0.6886$) in the probands. A second haplotype (GCAC) approached significant effect with BMI ($\beta = 0.18$; $p = 0.0557$) in the probands but did not significantly affect plasma creatinine ($\beta = -0.04$; $p = 0.7230$), GFR ($\beta = 0.07$; $p = 0.5340$), urine ACR ($\beta = -0.08$; $p = 0.4970$) and serum leptin ($\beta = 0.07$; $p = 0.4040$).

In the relatives of the probands (Table 8.16b), the GAAC haplotype (compared to the base haplotype GCAC) was detected to significantly increase urine ACR ($\beta = 0.71$; $p = 0.0437$), but had no significant effect on other phenotypes of renal function: plasma creatinine ($\beta = -0.21$; $p = 0.4650$), GFR ($\beta = 0.22$; $p = 0.4930$), or phenotypes of obesity: BMI ($\beta = -0.36$; $p = 0.2763$) and serum leptin ($\beta = -0.24$; $p = 0.3700$). However, GCAT had a significant effect on BMI ($\beta = -0.99$; $p = 0.0346$) and was observed to increase urine ACR ($\beta = 1.35$; $p = 0.0743$),

however, this effect on urine ACR did not reach significance. Other haplotypes did not show any effect on these phenotypes (Tables 8.16a and 8.16b).

Linkage disequilibrium was observed between G2548A and A19G (D' = 0.3767; χ^2 = 4.21; p = 0.0401), C188A and A19G (D' = 0.9028; χ^2 = 29.30; p < 0.0001) and A19G and C538T (D' = 0.9987; χ^2 = 26.92; p < 0.0001) in probands (Figure 8.2a). In the relatives of probands (Figure 8.2b), LD was observed only between G2548A and A19G (D' = 0.9966; χ^2 = 6.69; p = 0.0096) and between C188A and A19G (D' = 0.9986; χ^2 = 7.94; p = 0.0048) (Figure 8.2a and 8.2b).

Table 8.15: Comparison of haplotype frequencies between probands and their first degree relatives.

G2548A	C188A	A19G	C538T	Haplotype frequency		
				Total	Probands	Relatives
G	C	A	C	0.39	0.37	0.46
G	C	A	T	0.06	0.06	0.03
G	A	A	C	0.08	0.08	0.07
G	C	G	C	0.41	0.42	0.36
A	C	A	C	0.02	0.03	0.00
A	C	G	C	0.04	0.04	0.05

Figure 8.1: Inferred haplotype frequencies in the study population.

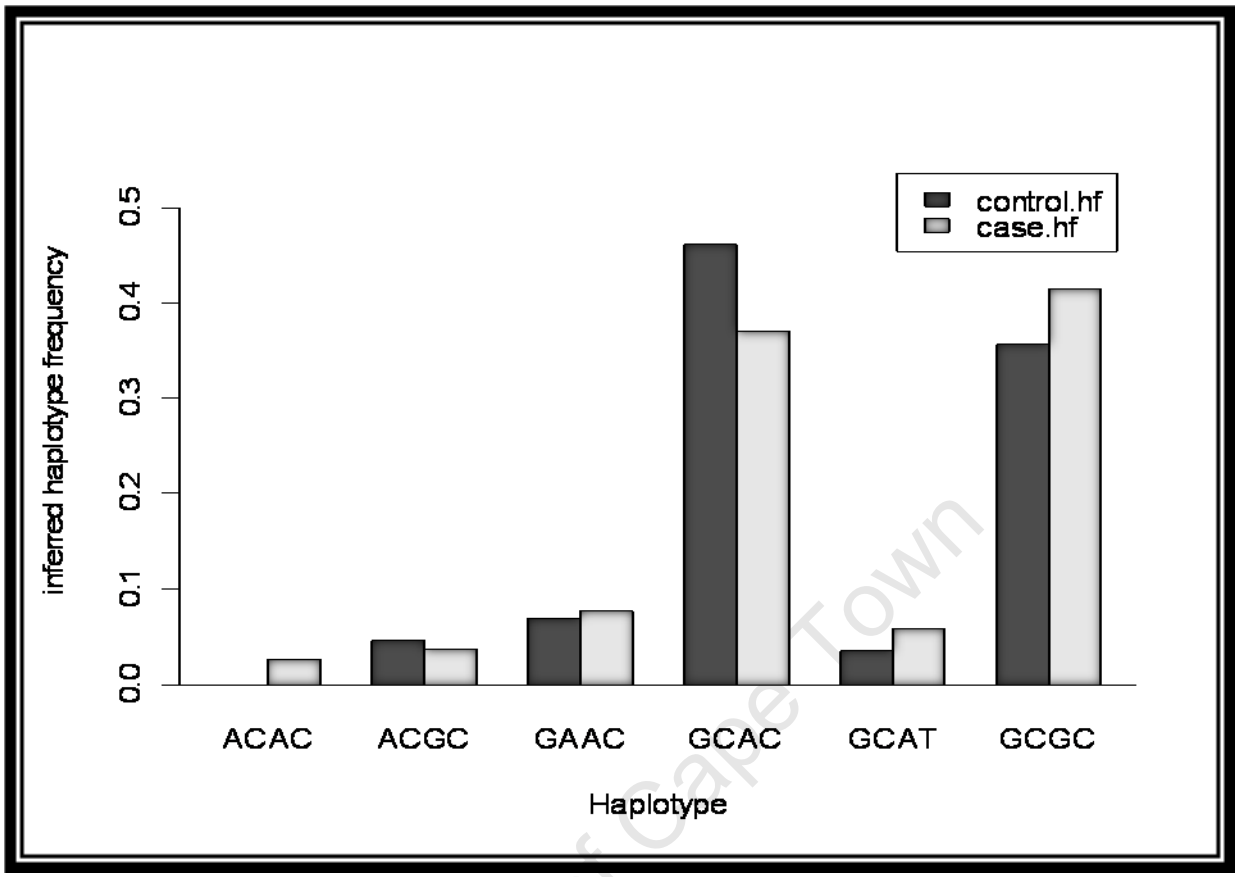


Table 8.16a. Test of association of haplotypes with phenotypes of interest in probands.

G2548A	C188A	A19G	C538T	Haplotype Frequency	PCr		GFR		ACR		BMI		Leptin	
					β	p	β	p	β	p	β	p	β	p
A	C	A	C	0.03	0.29	0.3750	-0.41	0.1760	-0.03	0.9210	0.31	0.2519	0.25	0.3695
A	C	G	C	0.04	-0.12	0.7160	0.21	0.5210	-0.16	0.6490	0.29	0.2532	-0.10	0.6750
G	A	A	C	0.08	0.00	0.9870	-0.06	0.7420	-0.29	0.1480	0.12	0.4313	0.15	0.3297
G	C	A	C	0.37	-0.04	0.7230	0.07	0.5340	-0.08	0.4970	0.18	0.0557	0.07	0.4040
G	C	A	T	0.06	-0.41	0.0279	0.46	0.0173	-0.12	0.5960	0.35	0.0514	0.07	0.6886
G	C	G	C	0.42	**Base haplotype**									

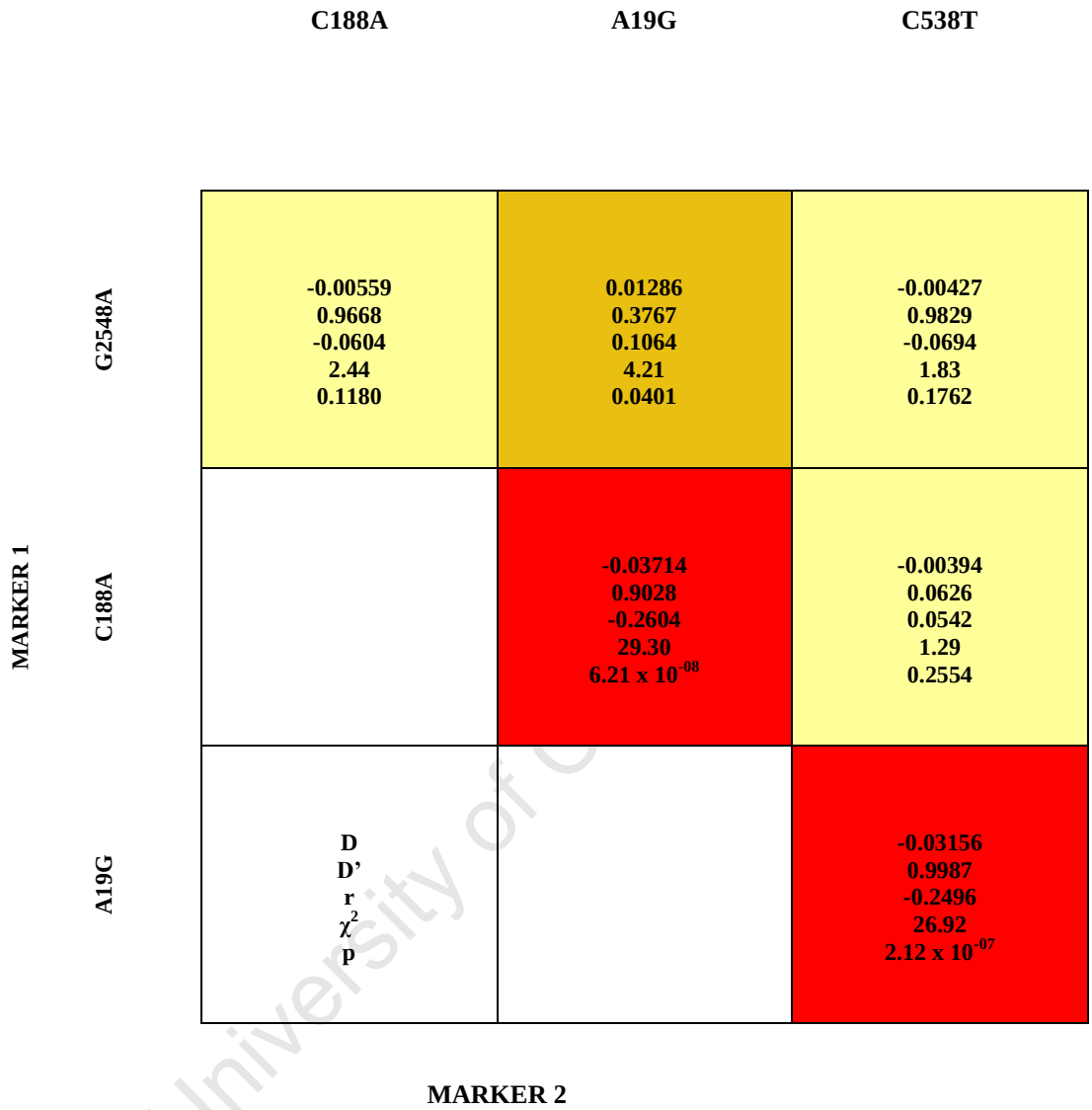
Tests are adjusted for age and sex.
β = estimated difference in transformed phenotype between individuals with a given haplotype and individuals with the base haplotype.
Pcr = plasma creatinine, GFR = Glomerular filtration rate, ACR = Albumin-to-creatinine ratio, BMI = Body mass index,

Table 8.16b. Test of association of haplotypes with phenotypes of interest in relatives of probands.

G2548A	C188A	A19G	C538T	Hap Freq	PCr		GFR		ACR		BMI		Leptin	
					β	p	β	p	β	p	β	p	β	p
A	C	G	C	0.05	-0.11	0.7650	0.29	0.4920	0.18	0.6835	0.46	0.3179	-0.34	0.3330
G	A	A	C	0.07	-0.21	0.4650	0.22	0.4930	0.71	0.0437	-0.36	0.2763	-0.24	0.3700
G	C	A	T	0.02	-0.28	0.4790	0.45	0.4820	1.35	0.0743	-0.99	0.0346	-0.50	0.2020
G	C	G	C	0.34	-0.01	0.9480	0.12	0.5380	0.19	0.3194	0.26	0.1872	0.05	0.7470
G	C	G	T	0.05	-0.34	0.4540	0.47	0.3000	-0.18	0.6845	-0.15	0.7930	-0.05	0.9010
G	C	A	C	0.48	**Base haplotype**									

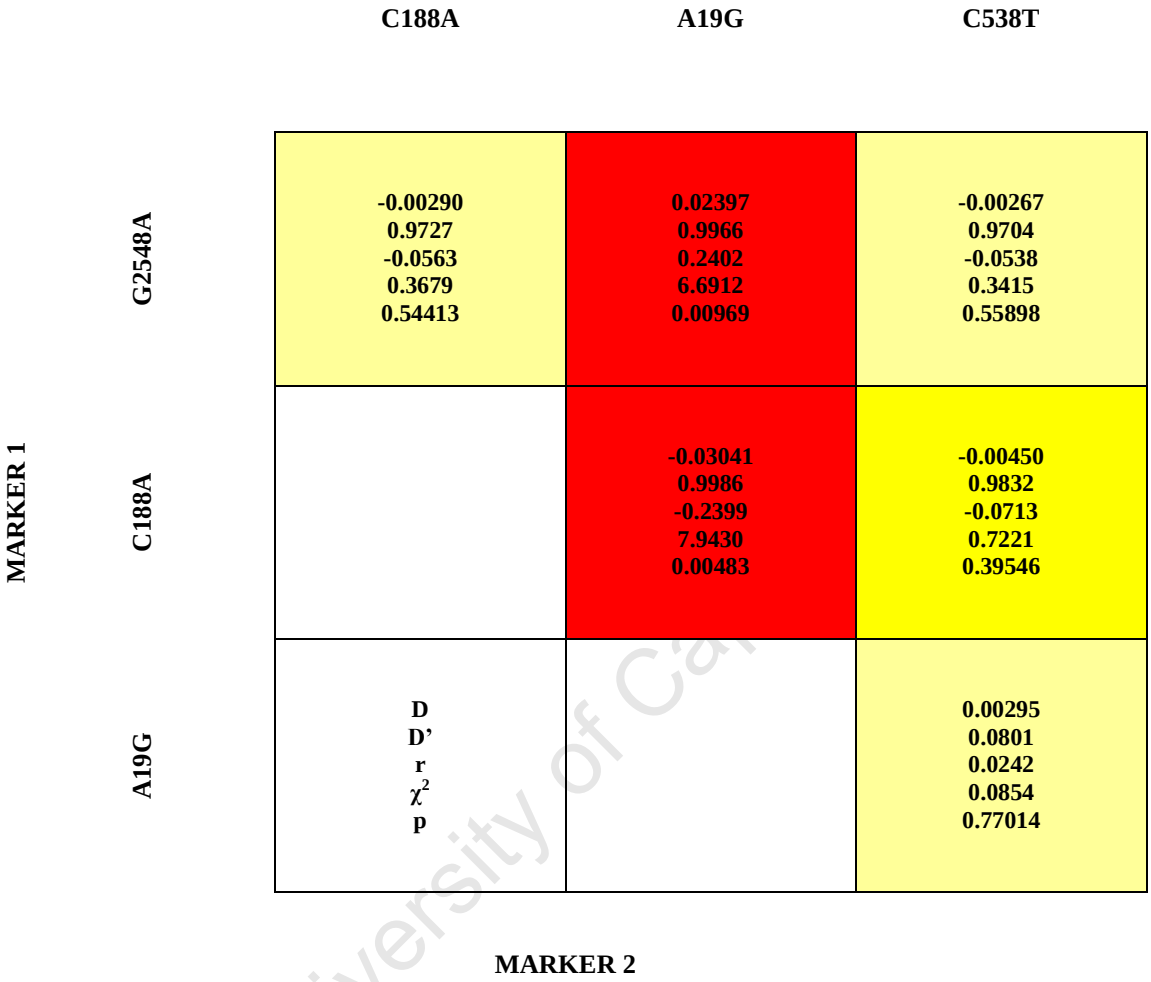
Tests are adjusted for age and sex.
β = estimated difference in transformed phenotype between individuals with a given haplotype and individuals with the base haplotype.
Pcr = plasma creatinine, GFR = Glomerular filtration rate, ACR = Albumin-to-creatinine ratio, BMI = Body mass index,

Figure 8.2a: LD plot of the probands



The darker colours show stronger LD

Figure 8.2b: LD plot of the relatives of probands



The darker colours show stronger LD

8.4 DISCUSSION

8.4.1: Genotype – phenotype Association:

Leptin is the obesity gene product secreted by white adipose tissue. Serum leptin concentration increases proportionately with degree of fat mass or obesity and in obese humans, leptin exerts a deteriorative impact on the cardiovascular system and the kidneys by significant contribution to the pathogenesis of obesity-related hypertension.

A number of studies (referenced in chapter 5 and below) have evaluated the effect of the LEP gene on CVD phenotypes like obesity often using BMI as the phenotype for obesity. Results from these studies have largely remained controversial with some studies reporting association and others reporting a lack of association. Few studies have studied the relationship between the LEP gene and CVD using markers other than BMI to represent CVD.

In this study, the relationship between 4 non-coding SNPs (G2548A, C188A, A19G and C538T) spanning the different haplotypes of the LEP gene and CVD markers (obesity, the MS, hypertension and microalbuminuria) was explored in a non-diabetic native black (South) African population that included hypertensive parents and their normotensive first degree relatives.

Although serum leptin had been shown to be positively and significantly correlated with all the obesity phenotypes (see chapter 7), there was no significant association between these polymorphisms and the obesity phenotypes or serum leptin concentration (BMI of $\geq 30 kg/m^2$ is conventionally used to define obesity and was also used in this study). However, genotypes

of the polymorphisms in the promoter region of the gene (G2548A and C188A) showed a trend towards significance with regards to obesity. There were 14.6% obese subjects whose genotype was GA at the 2548 position compared with 7.2% non-obese subjects with the same genotype ($p = 0.079$) and the subjects with this genotype had a higher mean BMI compared with subjects in the GG genotype group (Table 8.6). Also, using the co-dominant model in the analysis of the C188A genotype, there was no difference between the distribution of obese and non-obese subjects within the 3 genotypes (CC/CA/AA); $p = 0.194$ (Table 8.7.1). However, analysis using the dominant model (CC vs. CA + AA) revealed a trend towards significance ($p = 0.084$) in relationship with obesity.

Li *et al* (1999) reported significantly different frequencies in the distribution of the alleles of the A19G and G2548A polymorphisms between obese and average weight Caucasian women. Additionally, the A19G and C188A polymorphisms were found to predict the degree of obesity (Li *et al* 1999). Other studies that have reported association between obesity and LEP polymorphisms include those of Wang *et al* (2006), Jiang *et al* (2004), Hager *et al* (1998) and Mammes *et al*, (1998, 2000). These studies were carried out in Caucasian or Asian populations. However, there are other studies like those of Yiannakouris *et al* (2003), Shintani *et al* (2002), Lucatoni *et al* (2000) and Gaukrodger *et al* (2005); also carried out in European or Asian populations that have reported lack of association between LEP polymorphisms and obesity.

This study is the first in a homogenous black African population and as such provides reference data for this population. Beyond this however, it has shown that the common polymorphisms of the LEP gene are not significantly associated with obesity or increased serum leptin concentration in black Africans. This finding might imply that the LEP gene

plays no role in the pathogenesis of common obesity occurring in many South African blacks. However, although these polymorphisms have shown no association with serum leptin concentration, it still does not rule out their possible role in the expression of the gene, conformation and structure of the gene product (not tested in this study) which might ultimately influence its function peripherally.

It is thought that essential hypertension and obesity are both caused by multiple environmental and genetic determinants. Studies have shown that these two traits are linked and it is estimated that essential hypertension may be up to 3 times more prevalent among the obese (Van Itallie 1985). This correlation suggests that essential hypertension and obesity may share genetic determinants in some individuals. The cardiovascular effects of leptin have been discussed in chapter 5 and this study has already shown the increasing risk for kidney disease with increase in serum leptin concentration (figure 7.1).

There are only a few studies that have studied the relationship between the LEP gene and CVD. For instance, Shintani *et al* (2002) reported association between a polymorphic tetranucleotide repeat $(TTTC)_n$ polymorphism in the 3' flanking region of the LEP gene and hypertension in a group of Japanese patients with essential hypertension. They found the frequency of the class I allele to be significantly higher in hypertensives compared with normotensive controls (Shintani *et al* 2002). In two other different studies in which the same polymorphism was examined in South Americans and Italians, there was no association between the class I / II genotypes or alleles with hypertension or CVD (Hinuy *et al*. 2006, Porreca *et al*. 2006).

Gaukrodger *et al* (2005) reported an association between the rare C538T polymorphism in the non-coding region of exon 3 of the LEP gene with atherosclerosis. Their study has so far had the largest number of subjects in studies related to the obesity gene with a total of 1428 United Kingdom participants. They found a strong association between the LEP C538T and both pulse pressure ($p = 0.0001$) and carotid intima-media thickness (CIMT), a surrogate marker of atherosclerosis ($p = 0.008$). They also observed that subjects with the C/T genotype had a 22% lower pulse pressure and a 17% lower CIMT than C/C homozygotes suggesting that the presence of the T allele is "protective" for CVDs.

In my study, although there has been no segregation of any of the genotypes or alleles with any particular phenotype assessed, it was observed that certain genotypes in 2 of the polymorphisms (A19G and C538T) showed significant relationship with microalbuminuria, a surrogate marker of endothelial dysfunction and CVD.

Subjects with the AA genotype of the A19G polymorphism had a significantly higher mean urine ACR compared with genotypes AG and GG (3.7 ± 1.1; 2.4 ± 0.4; 1.4 ± 0.3; respectively, $p = 0.049$), a trend that appeared to show that ACR increased with an increasing presence of the A allele (Table 8.4.1). Also, subjects with the genotype AA, had the highest odds ratio for developing microalbuminuria, almost thrice that of subjects whose genotype was GG (Table 8.5)

All the offspring (normotensive, non-diabetic, and non-obese) were analysed separately to study if the same genetic trend had been passed on to them (Table 8.4.2). Again, subjects with the AA genotype had a significantly higher mean ACR in comparison with those of the AG or GG genotypes (5.7 ± 4.0; 0.8 ± 0.3; 2.0 ± 0.9 respectively; $p = 0.026$). Although estimated

GFR was not significantly different between all the groups, GFR always was lowest in the AA genotype group compared with the other groups suggesting a poorer renal outcome in this group (Tables 8.4.1 and 8.4.2).

Furthermore, although both genotypes of the C538T polymorphism showed no difference in frequency between groups divided along the lines of CVD (present or absent), subjects with the CT genotype had significantly lower ACR compared with those with genotype CC (1.3 ± 0.2 vs. 2.7 ± 0.4; p = 0.001) (Table 8.12). The same trend was seen with the estimated GFR and when the subjects were analysed separately as probands and offspring (Table 8.13). Moreover, subjects with the CT genotype had a lower odds ratio for microalbuminuria compared with those with genotype CC. This pattern is similar to the earlier report of Gaukrodger *et al*. (2005) in which they found the T allele to be protective of CVD measured by atherosclerosis and defined by the thickness of the CIMT (Gaukrodger *et al* 2005).

Although it cannot be said that there is significant difference in the distribution of these genotypes of the LEP gene by the presence or absence of markers of kidney disease in this population, the present trend with the genotypes of the A19G and C538T polymorphisms suggest that there may be some relationship between the LEP gene and kidney disease, especially since serum leptin has earlier been shown to correlate with decreasing GFR in this same population (chapter 7). There are no reports in the literature of relationships between this gene and kidney disease phenotypes despite the relationship between the gene product, obesity and kidney disease that has also been shown by the present study. This association is important as it shows that obesity plays a significant role in kidney disease both phenotypically and genetically.

The leptin signalling pathways cross-talks with the insulin signalling pathways and although the molecular mechanisms of these pathways were not analysed in this current study, it is possible that the pathway leading to inflammation might be involved in the associations found between these polymorphisms and microalbuminuria. Therefore their effect on the kidney may be through an autocrine or paracrine mechanism. Also, even though the C538T polymorphism is in the non-coding 3' UTR region of the gene, similar variants in other genes have been reported to affect their expression (Misquitta *et al* 2001).

Although ESRD is quite common in people of African origin, there have often been questions as to why black Africans have a preponderance of hypertension-related ESRD. Differences in socio-economic status, higher prevalence of hypertension and an increased inherited susceptibility of black Africans to kidney disease have been suggested as explanations to account for these differences (Seedat 1999, Krop *et al* 1999). This thesis has shown that genetic factors such as the polymorphisms of the obesity gene contribute significantly to kidney disease patterns in black Africans.

By whatever criteria the MS is defined, its presence can only be confirmed by the presence of multiple metabolic risk factors, as at least the combination of three or more risk factors out of a possible five are required to make the diagnosis. Studies that have reported association of candidate genes with the MS have often done so with specific genes related to one of the MS factors. For instance, Lee and Tsai (2002) reported association between the ACE I/D polymorphism and the 1998 WHO definition of the MS in Chinese NIDDM patients while Frederiksen *et al* (2002) also found an association between a polymorphism of the PPAR-gamma gene and the MS in Danish MONICA subjects.

In chapter 3 of this thesis, the mechanism of the MS was traced through the obesity pathway and was shown to be an inflammatory disease triggered by the presence of excess circulating fatty acids that ultimately result in the secretion of several pro-inflammatory cytokines. Although the relationship between obesity and the MS is very clear, Meirhaeghe *et al* (2005) found no association between a polymorphism of the obesity gene (LEP A19G) and the MS in Europeans. However, the National Heart, Lung, and Blood Institute (NHLBI) Family Heart Study on linkage analysis of a composite factor for the MS detected 2 regions on chromosome 6 (for a factor loaded by BMI, leptin and fasting insulin) and chromosome 7 (for a lipid factor) with strong evidence for linkage to the MS (Tang *et al* 2003). In the NHLBI study, the region on chromosome 7 with suggestive evidence of linkage with the MS contained the leptin gene.

Finally, although no association between the LEP gene and CVD marker has been seen in this study, the observed trend with the A19G and C538T polymorphisms with microalbuminuria suggests that an association indeed exists as the gene product has been shown to correlate with GFR in chapter 7.

These findings do not rule out the importance of genetics or indeed the LEP gene in complex diseases such as obesity or the MS. However, in this specific population of native black Africans, the LEP gene has been shown to have no effect on complex disease phenotypes like hypertension, obesity and the MS, but may play a role in the pathogenesis of kidney diseases.

The power of the entire study was appropriately high (80%) to demonstrate statistically significant associations that could be of clinical importance. The study was not powered for sub-group analysis but the fact that some of the associations studied (A19G and C538T with

microalbuminuria) are of statistical significance provides reassurance regarding the validity of the data presented herein. That these results may not have been due to poor sampling is also shown through comparable sample sizes with previous similar studies (already referred to) from other population. Only one study has used a very large number of subjects and the results obtained from this present study has been comparable (Gaukrodger *et al*. 2005).

8.4.2: Haplotype Analysis:

Only 2 of the 4 SNPs typed in this study are present in the HapMap YRI sample. As such, it is not possible to directly compare the haplotype frequencies in the present study with the YRI HapMap haplotype frequencies which represent the HapMap data from Africa. However, the polymorphisms we typed at LEP captured all common haplotype variations across the gene. The six haplotypes I reported in this population are similar to the top 5 haplotypes reported in a European population in which association between the leptin gene and atherosclerosis was found (Gaukrodger *et al*. 2005). The haplotype frequencies in this population are also comparable to the frequencies reported from the European population (Gaukrodger *et al*. 2005).

My analysis identified two haplotypes that affected phenotypes of renal disease (in comparison to the base haplotypes) in this Xhosa population (GCAT and GAAC). The GCAT (frequency of 6%) appeared to protect against renal disease (decreasing plasma creatinine, increasing GFR and reducing urine ACR), while the GAAC haplotype (frequency of 8%) in the relatives was observed to have an increasing effect on urine ACR. This might suggest that an allele in the GCAT haplotype could have a protective effect on kidney disease phenotypes in this population. As I have already shown in Table 8.13 ("Analysis of LEP C538T Genotypes with markers of renal function in probands and their offspring") the CT genotype

of the C538T polymorphism had significantly lower urine ACR (p = 0.004) and higher GFR (p = 0.010) in the probands compared to the subjects with the CC genotype. This protective effect had also been reported in a different study among Europeans who carry the T allele of the C538T who were reported to have lower pulse pressure (p = 0.0001) and lower carotid intima-medial thickness (p = 0.0076) compared to those who did not carry this allele (Gaukrodger et al. 2005). The mechanism of this "protection", although at present not clear, does not seem to be dependent on variation in serum leptin concentration which was not affected by the presence of this haplotype.

The effect of the haplotype GAAC on urine ACR is in keeping with our finding from genotype analysis in which the "A" allele of the A19G polymorphism was found to be associated with markers of kidney disease in this population (Tables 8.4.1, 8.4.2 and 8.5). This may suggest that the "A" allele of A19G is a risk allele for kidney disease in this population. However, the presence of the T allele of C538T may probably mask the effect of this allele in the population. The mechanism of this relationship may, however, not be through the direct effects of serum leptin as there was no association between serum leptin and the haplotypes. This association may probably occur via an autocrine or paracrine effect of leptin due to the ubiquitous distribution of leptin receptors which underlies its pleiotrophic roles (Fruhbeck 2006). The paracrine loop will require the presence of a specific ligand-binding receptor on the target cell. Leptin receptors occur in multiple forms (see section 5.3); the long form (LRb) has an intracellular domain of sufficient length to provide for full signal-transducing capabilities. LRb can activate STAT factors 1, 3, and 6, MAPK protein (Baumann et al. 1996). Although a number of studies (Kraus et al. 2002; Karmazyn et al. 2007; Singhal et al. 2002) have suggested the autocrine and/or paracrine actions of leptin in various diseases, I can only assume that this may be the same mechanism through which the

renal phenotypes have been affected in our study. Nevertheless, our finding is consistent with a previous report that the chromosome 7 harbours a locus for increased susceptibility to kidney disease in black Africans (Chen *et al*. 2007, Placha *et al* 2005).

As my study design is confined to a single black African ethnic group (Xhosa) with a moderate sample size, the conclusion may not be pertinent to other races or ethnic groups and therefore replication of this study in other population may be required. However, the results obtained in this study from both genotype and haplotype analysis will suggest that the obesity gene plays some role in kidney disease in this population.

Consequently, as already alluded to, there may well be other genes that are supplementary to the phenotypic expression of complex diseases like kidney disease, obesity and the MS. In the next chapter, we explore what further candidate genes may be identified through computational methods and that may play a role in the pathogenesis of the MS since we have been unable to identify a role for the LEP gene in the MS in this chapter.

CHAPTER 9

COMPUTATIONAL ANALYSIS FOR METABOLIC SYNDROME GENES

As already stated in the acknowledgements section, the contents of this chapter represents collaborative work by Dr Nicki Tiffin (Bioinformaticist, Department of Human Molecular Genetics, UCT), Drs Miguel Andrade-Navarro and Carolina Perez-Iratxeta (Ontario Genomics Innovation Centre (OGIC), Ottawa Health Research Institute (OHRI) Ontario Canada and Department of Cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa Canada) and myself. The work of this chapter was conceived by Dr Tiffin and me and she implemented the study and prepared the manuscript. Miguel and Carolina specifically ran the scripts for Genes2Disease (G2D) and GeneOntology (GO) and helped with the phenotype analysis associated with this chapter and also contributed to the manuscript. I provided clinical input and analysis and contributed to the manuscript.

9.1 INTRODUCTION

Many diseases may be caused by loss or gain of gene function, and several genes have been implicated in Mendelian diseases. The methods which have been used to find Mendelian disease genes have often failed to identify genes central to complex diseases that often present with a wide range of phenotypes and generally involve multiple aetiological mechanisms and contributing genes (Glazier *et al*. 2002). In complex diseases, the contribution of each of several genes related to the disease state is likely to be small, and only the sum of several susceptibility genes leads to disease, making functional validation of complex disease-causing genes difficult through methods employed for Mendelian diseases (Hoh and Ott. 2004).

Also, the comprehensive analysis of so many genes generated through genome-wide techniques can be extensively time-consuming and expensive (Risch. 2000), for example, loci implicated in susceptibility to multi-factorial disease by linkage analysis can be up to 30 Mb, often containing several hundreds of genes (Perez-Iratxeta *et al*. 2002). Hence, computational methods embracing the so-called "bioinformatics approach" have an important role in the analysis of candidate genes and in the selection of the most likely of these genes to be experimentally confirmed in complex diseases, such as the MS.

A number of computational methods have been developed that search through many different data sources containing information on sequence, biology, function and expression of candidate genes (Kanehisa and Bork 2003). Here, we employ such methods to comprehend and select candidate genes that may play a role in the MS.

9.2 EXISTING COMPUTATIONAL METHODS

9.2.1 GENESEEKER (van Driel *et al*. 2005):
(http://www.cmbi.ru.nl/geneseeker/)

GeneSeeker is a web tool that filters positional candidate disease genes based on expression and phenotypic data from both human and mouse. Although it is particularly suited for syndromes in which the disease gene shows altered expression patterns in the affected tissues, it can also be applied to complex diseases.

9.2.2 ANALYSIS OF CANDIDATE GENE EXPRESSION USING eVOC ANNOTATION (Kelso *et al*. 2003, Tiffin *et al*. 2005):
(http://www.sanbi.ac.za/resources/tools-for-downloading/)

This method performs candidate disease gene selection using a controlled and structured vocabulary for unifying gene expression data (the eVOC anatomy ontology) i.e. candidate disease genes are selected according to their expression profiles, using the eVOC vocabulary

to integrate clinical and molecular data through a combination of text- and data-mining. It selects candidate genes according to their expression profiles within tissues associated with the disease of interest.

9.2.3 DISEASE GENE PREDICTION (DGP) (Lopez-Bigas *et al*. 2004):
(http://cgg.ebi.ac.uk/services/dgp/)

Genes already known to be involved in monogenic hereditary disease follow specific sequence property patterns that make them more likely to mutate. Based on these patterns, DGP is able to assign probabilities to all the genes that indicate their likelihood to mutate based on their sequence properties. Properties of the gene analysed include protein length and the degree of conservation. Although designed for the prediction of Mendelian diseases, it can also be used for the identification of complex-disease genes as it will identify those genes with higher likelihood of suffering mutations.

9.2.4 PROSPECTR and SUSPECTS (Adie *et al* 2006):
(http://www.genetics.med.ed.ac.uk/suspects/)
(http://www.genetics.med.ed.ac.uk/prospectr/)

It can be shown that genes implicated in disease share certain patterns of sequence based features like larger gene lengths and broader conservation through evolution. PROSPECTR is an alternating decision tree programmed to use a scoring system to differentiate between the likelihood of a gene involvement in disease. It uses basic sequence information to classify genes and those that score over a certain threshold are classified as likely to be involved in some form of human hereditary disease while genes with scores under that threshold are classified as unlikely to be involved in disease. SUSPECTS then builds on this by incorporating annotation data from Gene Ontology (GO), InterPro and expression libraries and then ranks the genes according to the likelihood that they are involved in a particular disorder rather than human hereditary disease in general. The GO project

(www.geneontology.org) provides structured, controlled vocabularies and classifications that cover several domains of molecular and cellular biology and used in the annotation of genes, gene products and sequences. Many model organism databases and genome annotation groups use the GO and contribute their annotation sets to the GO resource (Harris *et al* 2004).

9.2.5 GENES TO DISEASE (G2D) (Perez-Iratxeta *et al*. 2005):
(http://www.ogic.ca/projects/g2d_2/)

This system scores all terms in GO according to their relevance to each disease starting from MEDLINE queries featuring the name of the disease. To identify candidate genes in a given chromosomal region, G2D performs BLASTX searches of the region against all the (GO annotated) genes in RefSeq.

9.2.6 POCUS (Turner *et al*. 2003):
(http://www.hgu.mrc.ac.uk/Users/Colin.Semple/)

POCUS exploits the tendency for genes predisposing to the same disease to have identifiable similarities, such as shared GO annotation, shared InterPro domains or a similar expression profile. Therefore where genes within different susceptibility regions for the same disease share GO or InterPro annotation and/or are co-expressed, these genes may be considered good candidates. Although genes may be selected as candidates on the basis of sharing only a single GO term or InterPro domain, genes lacking this annotation completely will not be selected.

9.2.7 ENDEAVOUR (Altschul *et al*. 1997):
(http://homes.esat.kuleuven.be/~bioiuser/endeavour/endeavour.php)

This method ranks candidate genes according to their similarity to a training set of genes (a set of data for which the outcome of a method is known; and can therefore be used to define the best parameters for that method). Endeavour accesses several online databases including

MEDLINE abstracts, Locus Link data, GO data, InterPro and Bind protein-protein interaction data, KEGG (Kyoto encyclopaedia of genes and genomes) pathway data, microarray and Expressed Sequence Tag (EST) -based data, transcription factor binding site (TFBS) cis-regulatory modules and sequence similarity by Basic Local Alignment Search Tool (BLAST), and returns a ranked list of candidates.

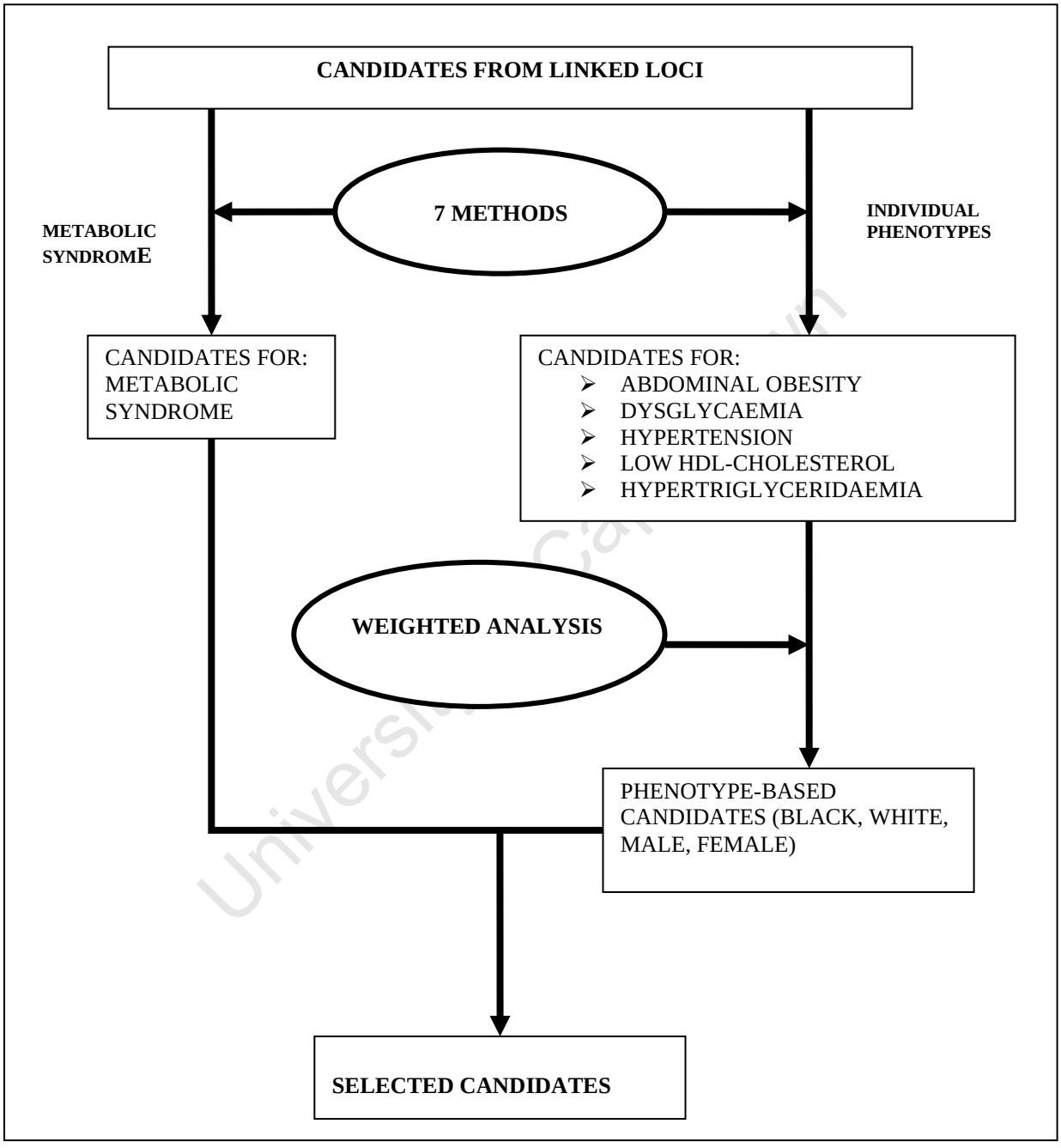
9.3 METHODOLOGY

An overview of the approach we used is shown in Figure 9.1. Multiple computational methods (described above) were used to analyse the starting set of candidate genes. The methods access an array of biological databases and analyse the candidates according to different criteria. Because of the large number of implicated loci, POCUS which is designed to analyse candidate genes lying in a few loci could not be used.

Candidate genes were scored according to the number of methods that select them. All the methods were independently used to select most likely candidates for MS and for candidates for each MS phenotype. Genes common to all the methods for the MS and for each phenotype were then selected.

Using a weighting system, genes selected for all the phenotypes were identified based on the frequency of occurrence of each phenotype in the presentation of MS. Data from Kraja *et al* (2005) and McCarthy *et al*. (2003) were used to repeat this analysis as they present frequencies of occurrence of these phenotypes in blacks and whites and in males and females respectively. Hence, the final candidate gene list consists of genes that are selected in the search for candidate genes for MS, and are also independently selected in the parallel selection of candidate genes for the five phenotypes in these four population groups.

Figure 9.1. Overview of analysis, showing selection of genes common to all methods, weighted selection of genes common to all phenotypes, and selection of genes common to MS and the phenotype analysis.



9.3.1 GENERATING THE STARTING SET OF CANDIDATE GENES:

Association studies for MS were used as a primary filter to select the starting set of candidate genes. Genes were compiled from cytogenetic loci using the Ensembl database Ensembl_mart_40 (www.ensembl.org) which provides a comprehensive and integrated source of annotation of chordate genome sequences (Hubbard *et al* 2007). The final candidate list comprised thirteen thousand, eight hundred and eighty-two (13 882) genes and covered all cytogenetic loci documented to be associated with the syndrome. Data for all genes was downloaded from the Ensembl database Ensembl_mart_41 into a local database for further analysis.

9.3.2 COMPILATION OF SEARCH TERMS AND TRAINING SETS OF DISEASE GENES:

Many of the methods described earlier require various user-defined inputs, and these are shown in table 9.1. These include disease search terms for querying abstracts in PubMed and anatomy terms used for GeneSeeker queries. Also shown are the top ranking eVOC terms selected by text-mining of PubMed abstracts containing the disease name. The MeSH terms used for each disease by G2D are as follows: for MS, all MeSH terms annotated in the literature references from the Online Mendelian Inheritance in Man (OMIM) entry 60552 were used. For its phenotypes; 'Obesity', 'Hypertension', 'Hypertriglyceridaemia', 'Diabetes Mellitus', and 'Cholesterol, HDL' were used.

The SUSPECTS software (Adie *et al*. 2006) automatically retrieves genes implicated in a disorder from OMIM database (McKusick. 2007), the Human Gene Mutation Database (Stenson *et al*. 2003) and Genetic Association Database (Becker *et al*. 2004). These training sets were used for each of the phenotypes, using the search terms outlined in table 9.1. For 'dysglycemia', no associated genes were found and a training set of 'diabetes'-associated

genes was used, as this is a very closely related condition. For ‘low HDL cholesterol’ no associated genes were found and ‘HDL cholesterol’-associated genes were used for the training set. Very few genes are associated with MS, so the training set was compiled from a set of MS-associated genes described by Matsunaga and Muramatsu (2005) which were derived by a sophisticated knowledge-based analysis involving text-mining of PubMed abstracts.

Table 9.1. Summary of search terms and training sets for MS and associated phenotypes. Query terms used to search biomedical literature for disease-relevant abstracts, and terms identified by text mining as most commonly associated with disease/phenotype (eVOC method) are also shown.

Disease/ phenotype	Search terms for PubMed	No of abstracts	Terms for GeneSeeker	Top eVOC terms	SUSPECTS and Endeavour training genes,
Abdominal obesity	"abdominal obesity", "increased waist circumference"	1098	adipose, kidney, heart, arteries, intestine	'cardiovascular|vascular', 'blood|peripheral blood', 'heart', 'oral|oral cavity'	"Abdominal Obesity" NR3C1, LPL,ADRA2A
Hypertri-glyceridemia	"hypertriglyceridemia", "hypertriglyceridaemia", "elevated triglycerides"	5543	adipose, liver, intestine, arteries	'cardiovascular|vascular', 'blood|peripheral blood', 'heart','hepatocellular|liver'	"Hypertriglyceridemia" APOA1, APOC3
Low HDL cholesterol	"low HDL cholesterol"	755	adipose, liver, intestine, arteries	'cardiovascular|vascular', 'heart', 'blood|peripheral blood', 'artery'	'HDL cholesterol' PPARG, GHR, LIPG, CETP, SCARB1, ABCA1
Hypertension	"hypertension", "high blood pressure"	Most recent 5000	kidney, arteries, heart	'cardiovascular|vascular', 'blood|peripheral blood', 'heart', 'artery'	"Hypertension" AGT, PNMT, AGTR1, GNB3, HSD11B2, NPR3, NOS3, SAH, CYP11B2, GNAS, TNF, LEPROT, ADRB2, NOS2A, RETN, TRHR, BDKRB2, ADM, EDNRA, CAT, DRD1, EDN2, APOE, HSD3B1, NPR1, NR3C2, ADD1, SCNN1B, SCNN1A, PPARG, PTGIS
Dysglycemia	"impaired fasting glucose", "dysglycaemia", "dysglycemia", "diabetes"	Most recent 5000	pancreas, liver, kidney, muscle, adipose, intestine	'cardiovascular|vascular', 'blood|peripheral blood', 'heart', 'oral|oral cavity'	"Diabetes" IRS1, TCF1, INSR, INS, AQP2, AVPR2, SLC2A4, AVP, HNF4A, SLC2A2, GCGR, GPD2, GYS1, HLA-DQB3, UCP3, PPARG, KCNJ11, IL6, IFNG, CYP1A1, CTLA4, BCHE, ADRB2, VDR, RETN, PAX4, CRP, ICAM1, PTPN22, IL18, TAP2, NOS2A, SUMO4, GH1, TCF2, IPF1, ADIPOQ, KCNJ9, IRS2, HK2, ISL1, FABP3, GCK, SORBS1, CAPN10, NOS3, FOXA2, MAPK8IP1, CP, GIPR, FOXP3, PTEN, CD38, AGER, STX1A, ABCC8, IAPP, TCF7, CPE, NEUROD1, AGRP, DCN, PPARA, UCP1, WFS1, ATP2A3, RXRG, PTGS2, LIPE
Metabolic syndrome	"metabolic syndrome"	5132	adipose, kidney, pancreas	'cardiovascular|vascular', 'blood|peripheral blood', 'heart', 'hepatocellular|liver'	"metabolic syndrome" ACE, LMNA, genes identified by Matsunaga *et al*. (24)

9.3.3 COLLATION OF GENE LISTS AND CONSTRUCTION OF A SCORING MATRIX:

Gene lists were analyzed using Microsoft Excel^{TM}. Visual Basic software, written to compile the data, assign scores to the candidates, generate final scores for candidates, and graphically present and rank them. Because the databases accessed by the various methods may have slightly different content, not all methods will return a result for all genes. If a method returns the gene as a likely candidate ('positive'), that candidate is assigned a score of 1. If the method returns the gene as an unlikely candidate ('negative'), the gene is assigned a score of 0. If the method does not analyze the gene ('null'), the score assigned is 0.5. Therefore, a gene that is not considered by a method has a marginally better ranking than a gene that is actively excluded by that method. The frequency of occurrence of individual MS phenotype is used for each population group (black, white, male and female) and the following scoring system is used: genes selected for each phenotype are given a value of the frequency of occurrence of that phenotype within the population. The frequency values of the phenotypes are shown in table 9.2.

For each gene, a final score is calculated as the sum of all scores assigned to it for that population. Final gene selections for each population are those genes selected for at least three of the five phenotypes (which are in general the highest scoring genes), and also any additional genes which score higher than the lowest score generated by the genes that are common to three of the five phenotypes.

Table 9.2. Phenotype frequency (%) described for four populations of MS patients.

POPULATION (reference)	Abdominal obesity (%)	Dysglycemia (%)	Low HDL cholesterol (%)	Hypertension (%)	Hyper-triglyceridemia (%)
Black (Kraja *et al* 2005)	63.66	23.88	40.43	66.55	16.35
White (Kraja *et al* 2005)	56.5	17.34	49.37	56.98	41.82
Male (McCarthy *et al*. 2003)	36	33	62	59	68
Female (McCarthy *et al*. 2003)	56	33	72	55	63

9.3.4 AUTOMATED ANALYSIS OF FINAL GENE LIST:

Enrichment for specific regulatory pathways and functional annotation was investigated using The Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/) (Dennis *et al*. 2003); and the reaction pathway database REACTOME (www.reactome.org), (Vastrik *et al*. 2007). Additionally, GOstat was used (http://gostat.wehi.edu.au/), (Beissbarth and Speed. 2004) to measure overrepresentation of Gene Ontology annotations (http://www.geneontology.org), (Harris *et al*. 2004) in the final candidate gene list.

In order to identify known relationships between the candidates described in the biomedical literature, the CHILIBOT data-mining software was used (www.chilibot.net) (Chen and Sharp. 2004), and the results obtained using CHILIBOT were manually curated for accuracy. Additionally the genes were investigated for known relationships to MS using CHILIBOT. Known or inferred protein-protein interactions between the candidate genes were investigated using STRING – the Search Tool for Retrieval of Interacting Proteins (http://string.embl.de); (von Mering *et al*. 2007). The interactions identified using STRING were manually curated for sufficiency of evidence.

9.4 RESULTS:

The numbers of genes selected by each method for MS and its five defining phenotypes are shown in table 9.3. The numbers of genes selected by all methods for each phenotype are shown in table 9.4, and those in common to both MS and all phenotypes, appropriately weighted according to frequency of occurrence of each phenotype, are also shown.

For the MS, 54 genes were selected by all methods used while for each of the phenotypes, the following numbers of genes selected were common to all methods: abdominal obesity – 48; dysglycemia - 58; hypertension - 69; hypertriglyceridemia - 80; low HDL cholesterol - 14. Genes were then scored according to their selection for the individual phenotypes in a weighted system whereby genes for more common phenotypes received higher scores. Using the phenotype frequency weightings, the following numbers of top-scoring genes were selected for each population: white population – 34 (score >113), black population – 41 (score >103), males – 36 (score >128), females – 34 (score >144).

There were 34 genes common to all these analyses, and of these 19 were also common to the set selected for MS (Appendix A5). These 19 were investigated further as a final candidate gene list. The genes selected using population-based weighting were compared to a selection made without weighting, i.e. all genes that appeared in at least three of the phenotype-specific sets were selected regardless of phenotype frequency. There were 34 genes that appeared in three or more phenotype sets, and these were the same 34 as for the white and female population-weighted data. However, six of these genes did not appear in the list selected using black population data. Seven additional unique genes appeared in the black population data set, and two additional unique genes were selected using the male population data. This illustrates the extent to which population-specific frequency data affects the final set of selected candidate genes.

Table 9.3. Number of genes selected by each method for MS and implicated phenotypes.

	PHENOTYPE					
Method	Metabolic Syndrome	Abdominal obesity	Dysglycaemia	Low HDL cholesterol	Hypertension	Hyper-triglyceridaemia
DGP	3034	3034	3034	3034	3034	3034
PROSPECTR	4287	4287	4287	4287	4287	4287
eVOC	4342	3584	3584	3572	3571	4342
GeneSeeker	959	1079	1263	1009	1032	1009
SUSPECTS	1896	2422	1836	2409	1841	2301
G2D	2369	2346	2473	145	2481	2446
ENDEAVOUR	2607	2607	2607	2607	2607	2607
Overlapping set:	**54**	**48**	**58**	**14**	**69**	**80**

Table 9.4. Number of genes selected for MS, for each phenotype, and in common to the selected sets (starting set 13,882 genes). Number of genes selected with phenotype weighting is shown for white/black phenotype distribution (Kraja *et al*. 2005) and male/female phenotype distribution (McCarthy *et al*. 2003).

	PHENOTYPE					
	Abdominal obesity	Dysglycemia	Low HDL cholesterol	Hypertension	Hypertriglyceridemia	Metabolic syndrome
Population	48	58	14	69	80	**54**
Black			41			
White			34			
Male			36			
Female			34			
Common genes:			**34**			
Common Genes:				**19***		

*Appendix A5 for the names and symbols of these 19 common genes.

9.4.1 CLUSTERING OF GENES BY FUNCTIONAL ANNOTATION USING DAVID:

The DAVID software analyses similarity of functional annotation of candidate genes, indicating the gene functions most commonly represented in the final candidate group of 19 genes. When using high stringency, the most enriched functional cluster, with a score of 3.9, contained annotation terms relating to lipid metabolism and transportation. The terms, shown with number of genes and p-values in parentheses, were "lipid transporter activity" (6; $p = 1.3 \times 10^{-8}$), "lipid metabolism" (5; $p = 6.0 \times 10^{-3}$), "transporter activity" (6; $p = 2.6 \times 10^{-2}$). The second cluster, with a score of 2.91, contained the terms, "signal" (10; $p = 5.9 \times 10^{-5}$), "signal peptide" (10; $p = 3.8 \times 10^{-3}$) and "disulphide bond" (9; $p = 8.7 \times 10^{-3}$). Using medium stringency generates a series of large, low-scoring term clusters that include terms involving lipid and chylomicron processing and transport, cell signalling, transmembrane/plasma membrane activity and tyrosine kinase activity.

9.4.2 ANALYSIS OF PATHWAYS INVOLVING THE CANDIDATE GENES USING REACTOME:

The SkyPainter utility available through the Reactome website highlights the pathways found most commonly associated within the final set of candidate genes. Of the starting set of 19 candidate genes, 9 were found in the Reactome database. The pathway most commonly represented is "Metabolism of lipids and lipoproteins" ($p = 6.2 \times 10^{-4}$). Processing of the chylomicron / apoE / LDLR complex is the most over-represented event, followed by LDLR/LDL processing.

9.4.3 ANALYSIS OF OVER-REPRESENTED GENE ONTOLOGY ANNOTATION OF CANDIDATE GENES USING GOSTAT:

The most representative GO terms for the final candidate gene list include terms relating to lipid transport and processing, signal transduction, plasma membrane location and development. The top-scoring fifteen terms, and the candidate genes that are annotated with those terms, are shown in table 9.5.

Table 9.5. Gene Ontology terms most overrepresented in the final candidate gene list, as determined using GOstat (http://gostat.wehi.edu.au/). Total number of candidate genes = 19, total number of annotated human genes used for statistical analysis = 33781. GO ID and corresponding term are shown, 'Count' is number of candidate genes with that annotation, 'Total' = total number of all genes with that annotation.

GO ID	GO Term	Genes	Count	Total	P-Value
GO:0005319	Lipid Transporter Activity	APOE, LDLR, APOB, HSD17B4, LPL, LRP1	6	87	3.23×10^{-9}
GO:0032501	Multicellular Organismal Process	APOE, INSR, LDLR, ITGB1, APOB, GIPC1, IGF1, LPL, PTPN11, TIE1, FYN, UTRN, FOXO1A, LRP1	14	3870	1.09×10^{-7}
GO:0042627	Chylomicron	APOE, APOB, LPL	3	8	1.40×10^{-6}
GO:0007275	Multicellular Organismal Development	APOE, INSR, ITGB1, IGF1, PTPN11, TIE1, UTRN, FYN, FOXO1A, LRP1,	10	2289	1.31×10^{-5}
GO:0007154	Cell Communication	APOE, INSR, AXL, ITGB1, APOB, GIPC1, IGF1, PTPN11, TIE1, FYN, ADCY7, FOXO1A, LRP1,	13	5554	6.38×10^{-5}

Table 9.5 (Contd)

GO ID	GO Term	Genes	Count	Total	P-Value
GO:0048731	System Development	APOE, INSR, TIE1, UTRN, FYN, FOXO1A, IGF1, PTPN11,	8	1623	$1.09x10^{-4}$
GO:0007165	Signal Transduction	INSR, AXL, ITGB1, APOB, GIPC1, IGF1, PTPN11, TIE1, FYN, ADCY7, FOXO1A, LRP1, ,	12	5125	$1.85x10^{-4}$
GO:0005887	Integral to Plasma Membrane	INSR, TIE1, AXL, ADCY7, LDLR, ITGB1, LRP1,	7	1358	$3.00x10^{-4}$
GO:0031226	Intrinsic to Plasma Membrane	INSR, TIE1, AXL, ADCY7, LDLR, ITGB1, LRP1	7	1370	0.0003
GO:0048856	Anatomical Structure Development	APOE, INSR, TIE1, UTRN, FYN, FOXO1A, IGF1, PTPN11	8	1990	0.0003
GO:0044459	Plasma Membrane Part	INSR, TIE1, AXL, UTRN, ADCY7, LDLR, ITGB1, LRP1	8	2149	0.000465
GO:0008201	Heparin Binding	APOE, APOB, LPL	3	78	0.000465
GO:0032502	Developmental Process	APOE, INSR, ITGB1, IGF1, PTPN11, TIE1, UTRN, FYN, FOXO1A, LRP1	10	3887	0.000532
GO:0007166	Cell Surface Receptor Linked Signal Transduction	INSR, FYN, ADCY7, ITGB1, FOXO1A, GIPC1, LRP1, PTPN11	8	2386	0.000812
GO:0004713	Protein Tyrosine-Kinase Activity	INSR, TIE1, AXL, FYN	4	323	0.000812

9.4.4 AUTOMATED LITERATURE ANALYSIS USING CHILIBOT:

Automated analysis of PubMed abstracts using CHILIBOT (www.chilibot.net), can detect existing associations reported between the disease, its phenotypes, and selected candidate genes, thus giving an indication of the degree of relevance and/or novelty of the selected candidates. The disease name "metabolic syndrome" was associated with 9/19 candidate gene names, and on inspection 8/19 were found to be real associations (APOB, APOE, FOXO1A, IGF1, INSR, LDLR, LPL and PTPN11 – the relationship indicated between 'metabolic syndrome' and CAT was spurious).

The nature of these associations is described in table 9.6. Potential indirect relationships with MS through one or more of the other short-listed candidates were indicated for all genes except ADCY7. Analysis of literature associating the candidate genes with each of the phenotypes indicates that only one of the candidates has been previously associated with all five phenotypes (APOB), five of the candidates have been associated with all phenotypes except dysglycaemia (IGF1, LDLR, APOE, INSR, LPL), and three of the candidates have not been independently associated with any of the phenotypes previously (ADCY7, GIPC1, PFKM).

Table 9.6. Genes previously associated with MS in the biomedical literature, as detected by Chilibot (www.chilibot.net). Common gene synonyms are shown in parentheses.

Gene	Summary of association with metabolic syndrome
APOB	The serum level of ApoB showed a strong correlation with MS in an Asian population (Moon *et al*. 2007).
APOE	Genetically obese Ay ApoE(-/-) knockout mouse model fails to develop obesity and MS features (Gao *et al*. 2007).
FOXO1	FoxO1 promotes insulin sensitivity and lipid synthesis in addition to glucose production. This dual role could explain the admixture of insulin resistance and sensitivity that is commonly observed in the MS (Matsumoto *et al*. 2006).
IGF1	Individuals with MS had lower IGF-1 levels than subjects without MS with an almost linear decline of IGF-1 levels as the number of fulfilled criteria of the MS (none to 5) increased (Efstratiadis *et al*. 2006).
INSR	A Muscle-Specific Insulin Receptor Knockout Exhibits Features of the MS of NIDDM (Hegele. 2003).
LDLR	Polymorphisms in LDLR were associatied with MS in coronary heart disease patients (LDLR_1 P =0.008; LDLR_2 P=0.018) (McCarthy *et al*. 2003). An LDLR knockout mouse is used as a model for MS (Seidelmann *et al*. 2005).
LPL	Decreased expression of LPL possibly causes the insulin resistance, in addition to hypertriglyceridemia, in MS (Shibasaki *et al*. 2006).
PTPN11 (SHP2)	Upregulation of cytosolic PTPN11 expression may contribute to the hemodynamic action as well as metabolic action in hypertensive MS (Wakino *et al*. 2004).

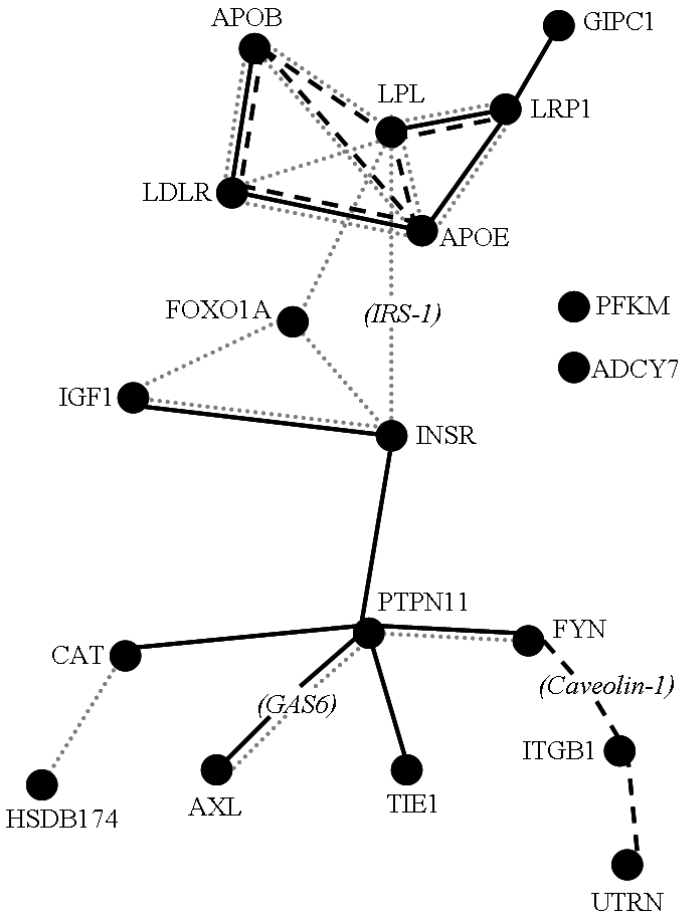
9.4.5 PROTEIN BINDING AND PROTEIN NETWORK ANALYSIS USING STRING:

STRING analysis indicates two main groups of interacting proteins within the final candidate list (figure 9.2.1). The first centres around PTPN11 (also called SHP2), and interactions between PTPN11 and FYN, TIE1, AXL, CAT and INSR (which in turn interacts with IGF1)

have been experimentally documented. These interactions are also described in the biomedical literature (PubMed abstracts) and in additional protein interaction databases (KEGG), which further extend the group by indicating interaction between ITGB1 and UTRN via FYN.

The second centres around LRP1, which interacts with GIPC1, LPL and APOE (which in turn interacts with LDLR and APOB). These interactions are also described in the biomedical literature and pathway databases. FOXO1A (also called FOXO1 and FKHR) lies between these two clusters, and a possible connection between the two clusters facilitated by this transcription factor is presented below. Proteins PFKM and ADCY7 are not included in the interacting groups.

Figure 9.2.1 Curated results from STRING analysis showing protein-protein interactions between the candidate genes (gene symbol shown at each node). Bold lines: experimental evidence; broken lines: evidence in pathway databases; grey dotted lines: evidence in the biomedical literature linking the two networks.

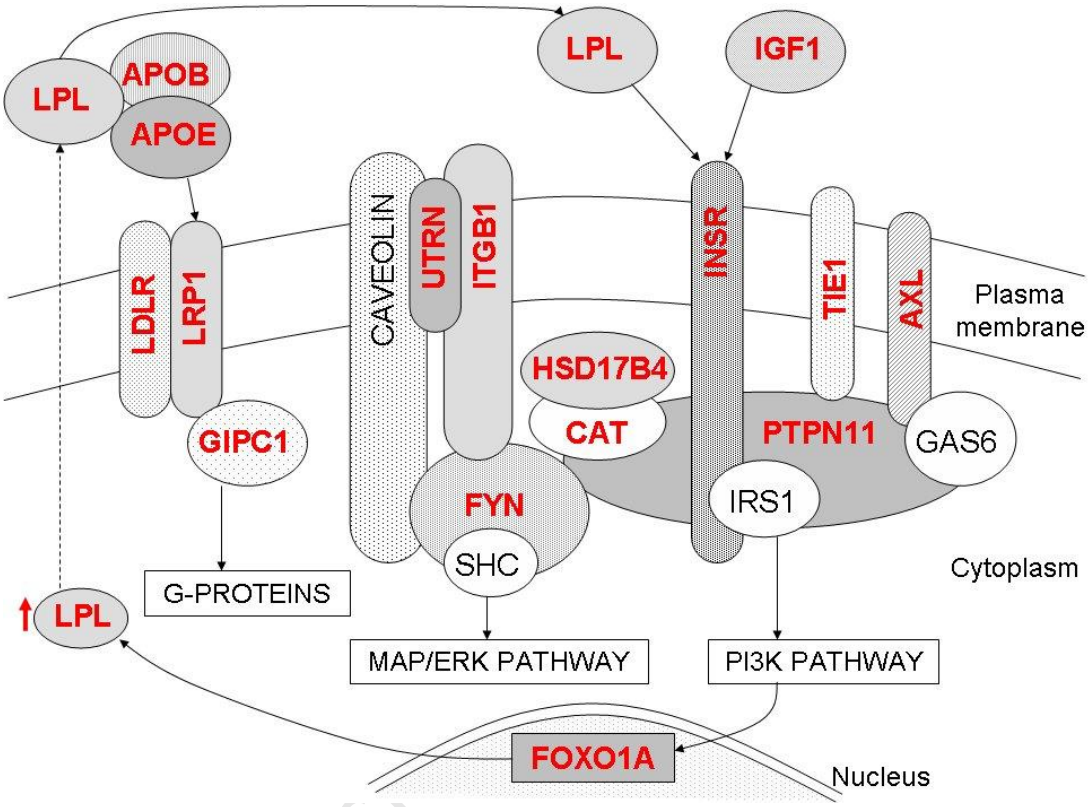


The potential interactions between the candidate genes' protein products are represented in figure 9.2.2, illustrating the predominance of genes in the final candidate set that are involved in chylomicron processing, transmembrane receptor activity and signal transduction.

Chylomicron processing is mediated by binding of an extracellular complex containing APOB, APOE and LPL to the transmembrane receptors LDLR and LRP1. The APOE and APOB components interact with LDLR; and the APOE and LPL components bind to LRP1 to facilitate chylomicron remnant uptake (Heeren *et al*. 2006). Three distinct complexes of transmembrane proteins can be identified in the candidate set. LDLR and LRP1 form one complex which binds GIPC1. UTRN, ITGB1 and FYN form a second (Wary *et al*. 1998).

Finally, INSR binds extracellular IGF1 as well as intracellular PTPN11 (also commonly called SHP2) (Asante-Appiah and Kennedy. 2003) to form the third complex. CAT and its binding partner HSD17B4, TIE1, FYN and AXL are also implicated in this third complex through their interactions with PTPN11 (Yano *et al*. 2004, van Grunsyen *et al*. 1998, Marron *et al*. 2000, Tang *et al*. 1999, Gallicchio *et al*. 2005). Additionally, the ability of FYN to interact with ITGB1/UTRN as well as INSR/PTPN11 suggests it may link these two complexes.

Figure 9.2.2 Potential binding interactions amongst candidate gene products, showing downstream signalling pathways activated. Candidate gene names are shown in **red**, additional proteins required for protein complexes are shown in black.



Three distinct signalling pathways are activated by genes in the final candidate set: The LDLR/LRP1 receptor complex activates G-protein signalling (Gotthardt *et al*. 2000); UTRN/ITGB1 in complex with FYN activates the MAP/ERK pathway and IGF1 binding to INSR activates the PI3K pathway (Kadowaki *et al*. 1996). LPL has also been implicated in INSR signalling, although the mechanism of this interaction is unclear (Shibasaki *et al*. 2006).

FOXO1A is the only nuclear transcription factor in the final candidate list, and is regulated via PTPN11 and IGF1/INSR binding and activation of the PI3K pathway (Schinner *et al*. 2005, Xu *et al*. 2004). FOXO1A can in turn upregulate expression of LPL (Kamei *et al*. 2003), an integral protein for chylomicron processing and a proposed regulator of insulin signalling (Shibasaki *et al*. 2006), thus providing a possible regulatory connection between transmembrane receptor activation/signal transduction, and the processes of chylomicron processing and insulin receptor signalling.

9.5 DISCUSSION:

We used computational methods to select the most likely candidate genes for MS, starting with a set of genes selected from all loci previously associated with the disease. The five phenotypes that define MS as well as phenotype and population-specific filters were used to refine the starting set of genes. The top-scoring 19 genes from the final candidate list were selected as most likely disease gene candidates. These candidates are found to most commonly represent pathways involving metabolism of lipids and lipoproteins, as well as transmembrane signalling and subsequent activation of signal transduction pathways.

Given the diversity of the five phenotypes associated with MS, and the variety of the phenotype combinations that present clinically, it would seem unlikely that genes belonging to specific and restricted pathways are causative of this syndrome. Instead, disease candidates are more likely to belong to non-specific pathways with multiple downstream effects, and thus be capable of generating a diverse array of phenotypic effects if disrupted. The predominance of receptor and signalling molecules in the candidate set fits this model: signal transduction pathways can have a large variety of initiating events and multiple downstream targets, regulated through a common signal transduction pathway. Additionally, because of the cascade effect in signal transduction events, even small changes in top-level signalling events can have large and diverse downstream effects – such as the variety of phenotypes seen in MS.

Of interest, the only transcription factor candidate, FOXO1A, appears to connect chylomicron remnant uptake with insulin signalling, and possibly functions in a feedback loop: FOXO1A is regulated by insulin signalling and upregulates LPL expression; and LPL in turn activates chylomicron processing and is involved in regulation of insulin signalling. Many of the genes

selected are also annotated with terms relating to development (Table 9.5). This most likely reflects a role for lipids and insulin in regulating genes involved in development (for example, neural development as described in (Gotthardt *et al*. 2000) rather than a role for developmental genes in MS - although this cannot be completely excluded, and these genes may have dual roles in metabolism as well as development.

Individually, eight of the candidates have been previously implicated in MS, and notably, polymorphisms in LDLR have been associated with MS previously (McCarthy *et al*. 2003). Only one of the candidates (APOB) has been previously associated with all five phenotypes. Three of the candidates show no association to these phenotypes, and one of these also has no direct or indirect association with MS (ADCY7) and is therefore a totally novel candidate. This selection of the top ranking 19 most likely candidates therefore presents an array of genes including a gene containing known disease-associated SNPs, some genes previously associated with MS and/or its phenotypes, and some entirely new candidates for further investigation. This demonstrates that novel predictions can be made from existing data using computational approaches, and that this approach is truly synthetic, and not simply sophisticated data-mining of existing knowledge.

Certain issues intrinsic to computational analysis however do arise. Firstly, differences between gene sets in different databases or versions of the same database was accommodated in part in this study by the scoring system, avoiding biased negative scoring against genes not measured by all methods. Secondly, genes with better annotation are generally more likely to be selected, resulting in a bias towards selection of well-investigated genes (including disease-related genes) rather than novel predicted or uncharacterised genes. Scoring by DGP, PROSPECTR, G2D and eVOC was applied to eliminate such biases. These methods employ

sequence similarity to analyse uncharacterised genes and are based on mapping of EST sequences from cDNA libraries regardless of the characterisation and annotation of the gene.

Thirdly, the use of training sets in selection limits the search to fit existing paradigms regarding the type of gene causing the disease, and limits the possibility of finding novel disease mechanisms. It would, however, be possible to investigate candidates selected without the use of training sets by excluding the methods that require a training set of known disease genes.

Finally, thresholds and cut-offs for selection of most likely candidates can be arbitrary or an 'informed guess' for methods that rank genes (in comparison to straightforward inclusion/exclusion). Where possible, thresholds in this study have been based on results from test datasets, as described in the seminal reference for each method.

Association studies have failed to date to identify causative genes underlying the syndrome, despite clear evidence for heritability (Pollex and Hegele. 2006), and this is most likely due to complex disease genetics whereby many aetiological genes of low penetrance, rather than a single causative gene, result in the syndrome. With multiple contributing phenotypes as well as many permutations of those phenotypes, the syndrome lends itself to being deconstructed for systematic computational analysis.

Here, the identification of "most likely" disease genes for MS has been addressed using computational rather than empirical methods. Results of published association studies have been used as a primary filter to select the starting set of candidate disease genes, and then

used multiple computational methods to select most likely candidate genes in a combinatorial approach informed by the clinical presentation of the syndrome.

Given the increasing urgency of understanding and managing MS effectively, it is shown here that computational analysis can offer new insights into genetic determinants and regulatory pathways that may be involved and presents a selection of genes that includes both novel and identified candidates for further empirical analysis. Exploring new avenues can also lead to a better understanding of environmental and genetic interactions underlying the syndrome.

CHAPTER 10

10.1 GENERAL DISCUSSIONS

Cardiovascular diseases constitute a major threat to the economies of many developing nations of the world. A number of these countries are confronted with a "double edged sword" situation in which they have to tackle epidemics of infectious diseases as well as those of NCDs. Kidney diseases requiring RRT for instance, require trained and dedicated health care workers, expensive machines and procedures, together with costly drugs, which most developing nations cannot afford, especially in the face of the need for measures to combat infectious diseases, poverty and hunger. Thus although the focus of several health care systems in Africa is often encouraged to revolve around communicable diseases like malaria, TB and HIV/AIDS, it is becoming increasingly clear that NCDs like obesity contribute significantly to morbidity and mortality in Africa.

As the obesity pandemic continues to gain momentum it will probably continue to profoundly affect mortality, morbidity, and quality of life worldwide unless measures are sought to address our ever increasing obesogenic environment. The MS, which is common and has been demonstrated to be prevalent in obese individuals, presents a prospect for seeking other factors (aside from hypertension alone) that potentially drive the increased prevalence of kidney disease in people of the black race.

This study has used the MS to investigate CVDs (using microalbuminuria as an entity that represents organ damage) in a homogenous population of native black Africans. The findings from this study have focussed on the phenotypic features and the genetic influence of the obesity gene on CVDs.

Although other studies have previously reported on the association of the MS and kidney disease, this study is the first of its kind in native black Africans, in whom it has been found that kidney disease, defined by the presence of microalbuminuria is found to be prevalent, with added metabolic risk factors such as the four-fold rise of ACR from subjects without MS phenotypes to those with 4 or more of the phenotypes and an increasing odds ratio for microalbuminuria with an increasing number of MS phenotypes (see chapter 7). Additionally, correlations between obesity phenotypes and markers of renal function have also been reported as has been in other studies (Chen *et al*. 2004; Bonneta *et al*. 2006). This study has also found serum leptin to be inversely and significantly correlated with markers of renal function, further implicating obesity specifically in the pathogenesis of kidney disease and more generally in CVDs.

Even though the exact mechanisms that link obesity and renal disease are still unknown, it has been suggested that some hormones secreted by fat cells (e.g. leptin, IL-6 and TNF-α) could be implicated in promoting renal damage (Wisse 2004). Leptin has previously been shown to be correlated with increased urine albumin excretion in women with type 1 diabetes melliuts (Rudberg and Persson 1998), suggesting a role in renal injury in this independent cohort.

An important question arising from this study pertains to the mechanism of kidney disease from a mutation occurring in the non-coding 5' UTR of the LEP gene. Our study showed that mean values of ACR was higher in subjects homozygous for the A allele of the A19G polymorphism (Chapter 8). This question is relevant because the effects on protein function of SNPs found in the 5' UTR region of the LEP gene have not been determined. Also, the 5'UTR region of any gene may influence translation of its down stream cistron (segment of DNA that is equivalent to a gene and that specifies a single functional unit) through its effects

on internal ribosome entry and the efficacy of translation of the downstream cistron (Pickering and Willis 2005).

The A19G polymorphism, together with 2 other SNPs (G2548A and C188A) analysed in this study are in the 5'UTR region of the LEP gene and therefore could affect the translation of the downstream cistron. The serum leptin concentration was not significantly different between the three genotype categories of the A19G polymorphism, however, protein function could conceivably be altered either by an abnormal folding pattern or conformation. This was not tested in the current study. Kimchi-Sarfaty *et al* (2007) have recently provided evidence that SNPs which have been assumed to be "silent" since they provide no change to the amino acid composition of the protein may indeed have significant effects on the protein function as such SNPs may alter the translation kinetics of the gene resulting in an abnormally folded and functioning protein (Kimchi-Sarfaty *et al* 2007).

In this thesis, bioinformatics methods have also been used to select the "most likely" candidate genes for the MS because the obesity gene didn't seem to be "it". The use of simple association methods underlying Mendelian diseases often fail to find genes responsible for complex diseases which usually occur from small contributions from several genes whose effects influence pathways related to the complex disease phenotype.

Here, the identification of "most likely" disease genes for metabolic syndrome has been addressed using computational rather than empirical methods. Results of published association studies have been used as a primary filter to select the starting set of candidate disease genes, and then used multiple computational methods to select most likely candidate genes in a combinatorial approach informed by the clinical presentation of the syndrome.

The top-scoring 19 candidate genes (See appendix A5) we reported are found to most commonly represent pathways involving metabolism of lipids and lipoproteins, as well as transmembrane signalling and subsequent activation of signal transduction pathways. That these genes represent pathways involving the metabolism of lipids and lipoproteins suggest that obesity is important in the pathogenesis of the MS. It also demonstrates that novel predictions can be made from existing data using computational approaches. With multiple contributing phenotypes as well as many permutations of those phenotypes, the syndrome lends itself to being deconstructed for systematic computational analysis.

Given the increasing urgency of understanding and managing MS effectively, it is shown here that computational analysis can offer new insights into genetic determinants and regulatory pathways that may be involved and presents a selection of genes that includes both novel and identified candidates for further empirical analysis. Exploring such new avenues can also lead to a better understanding of environmental and genetic interactions underlying the syndrome.

10.2 CONCLUSIONS AND RECOMMENDATIONS

The central theme of this study has been around obesity either manifesting as the MS or resulting in renal disease through the syndrome of insulin resistance. Certain conclusions can be drawn from the observations in this study:

1. The first is related to the principally observed phenotype results i.e. relationship between the MS and kidney disease. Although some have argued about the true relevance of the MS as a risk factor for CVD (Kahn *et al*. 2005), others have shown it is relevant in predicting CVD (Chen *et al*. 2004). This study has shown that such a relationship exists (with kidney disease) in non-diabetic subjects and underscores the importance of NCDs in populations of developing nations who are too selectively focused on the scourge of common communicable diseases.

2. This study has also shown that the LEP gene, in native black Africans, is associated with kidney disease measured by the presence of microalbuminuria. This therefore generates a novel hypothesis: that the LEP gene may be associated with kidney disease in non-diabetic black Africans and may need to be considered a candidate gene for kidney disease in black Africans. However, in view of small sample sizes in some of the analyses, this conclusion is to be taken cautiously and creates the need to replicate this finding in a similar population. The National Cancer Institute–National Human Genome Research Institute (NCI-NHGRI) working group have indeed suggested that replication studies need to be done on initial studies reporting an association (Chanock *et al*. 2007).

3. This study has found 8 out of 19 genes common to blacks and whites and males and females which have been linked to the MS and although these have not been experimentally

tested, it is proposed that empirical studies be carried out both in black and white populations to find a true association between the genes selected by computational methods and the MS and its phenotypes. This will strengthen the use of computational methods to select the "best" candidate genes underlying complex diseases.

Although, there have previously been reports from other population on the renal effects of the MS, this study is unique being the first to look for such an association in an indigenous black African population and having found such a relationship, corroborates previous reports from other populations. The novelty of this study also touches upon it being the first to report an association between the LEP gene and markers of kidney disease and in using computational methods to select candidate genes for the MS common to blacks and whites.

10.3 STRENGTHS AND LIMITATIONS OF THE STUDY.

There are a number of strengths and weaknesses identified in this study, summarized below.

STRENGTHS:

1. Focussing on the relationship between the leptin gene and renal disease phenotypes is a novel idea. This is important because there has been little empirical testing of this association, despite suggestive evidence in the literature. Investigating this issue within the context of the thesis therefore provides useful data as well as stimulating further studies.

2. The study of a population with no prior similar studies: black South Africans, is useful. In general, African populations are under-represented in such studies despite the useful insights that can be gained from them. Much of Africa is undergoing an epidemiologic and nutritional transition, providing an opportunity to evaluate what this means for complex diseases such as

hypertension, T2DM and the metabolic syndrome. The frequency of individual, behavioural and environmental risk factors for many complex diseases is lower and much more varied than in many Western countries, providing an opportunity to understand rural-urban differences and regional variation in Africa. African populations have the greatest genetic diversity of any continental populations and studies of African populations have the potential to exploit this diversity to identify disease associations and gain insights into biology.

3. A further strength of the study is that it represents an in-depth study of a large set of clearly-defined clinical and biochemical characteristics (i.e. a comprehensive study of a large number of variables in a modest number of study participants) rather than studying a few variables in thousands of subjects. This is clearly an advantage in exploratory studies such as this and the findings can then be used to design studies of more targeted variables in much larger samples.

4. The investigative use of bioinformatics techniques to provide additional insight into the potential genetic influences on the metabolic syndrome. As the volume and quality of data in the genetics and genomics-related databases have swollen in recent years, the use of computational biology techniques to guide, interpret or contextualize "wet-lab" experiments has become more and more important.

WEAKNESSES:

5. A modest sample size of three hundred and thirty-six subjects (336). While this was adequate for assessing the relationship between the metabolic syndrome an kidney disease in this population (upon which sample size estimation was based), it was insufficient for some of the analysis – like the association between the leptin genotypes and renal function phenotypes

in normotensive offspring of probands in which the total number was fifty (50) and those who were homozygous for the "A" allele were only six (6) subjects.

6. The cross-sectional nature of the study. Within cross-sectional studies, the 'exposure' and the 'outcomes' are usually assessed at the same time this hampers the interpretation of associations, as the data will usually not tell the investigator which came first: the 'exposure' or the 'outcomes' (Jager *et al* 2007). Therefore, this study design can draw limited inference with regard to relationships between exposure and outcome. It would have enhanced the study if the subjects were followed up for some period of time to determine phenotype end-points, for example the development of overt proteinuria – this was beyond the scope of this study due to time and patient availability constraints. This would clearly show, for instance, that subjects that started out with the MS and no microalbuminuria went on to develop full blown kidney disease much earlier than those without this condition or that normotensive, non-diabetic offspring of the probands who were homozygous AA for the LEP A19G polymorphism developed CKD much before hypertensive subjects without this genotype. A follow up would also help to determine if the GCAT haplotype protected those with hypertension or diabetes from developing kidney disease relative to blood pressure and glycaemic control. However, such a study would be very expensive and requires significant further time.

10.4 RECOMMENDATIONS

As in other genetic studies reporting association, a replication study is needed to confirm the results obtained from the present study. Future genetic studies should be adequately powered, especially for genetic analysis and should not be limited by the number of SNPs studied. Such studies may also examine the downstream effects of leptin on its signalling pathways and how the structure function of the protein is affected by the occurrence of certain mutations.

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APPENDICES
A1. PCR AND RESTRICTION ENZYME DIGESTS

A1.1 PCR ASSAY OF LEP A19G IN EXON 1 OF THE GENE

There are a number of studies that have reported about the A19G mutation (in text). These studies have done so with respect to obesity phenotypes and or serum leptin concentrations. Reports from the different populations have been non-uniform. I am presently unaware of reports of this mutation from studies conducted in any black African population.

The assay involves producing a fragment of 258 bp with 1 cutting site at position 186 of the fragment for the restriction enzyme MspA1I (cuts at 5'---C(A/C)G↓C(G/T)G---3' or 3'---G(T/G)C↑G(C/A)C---5'). A cut at this position (homozygote GG) will result in 2 bands (186 bp and 62 bp fragments). Heterozygotes thus display 3 bands: 258 bp, 186 bp and 62 bp fragments.

AMPLICON (258)

5' GCCCCGCGAGGTGCACACTGCGGGCCCAGGGCTAGCAGCCGCCCGGCACGTC
GCTACCCTGAGGGGCGGGGCGGGAGCTGGCGCTAGAAATGCGCCGGGGCCTGCG
GGGCAGTTGCGCAAGTTGTGATCGGGCCGCTATAAGAGGGGCGGGCAGGCATGG
AGCCCCGTAGGAATCGCAGCGCCAGCGGTTGCAAGGTAAGGCCCCGGCGCGCTC
CTTCCTCCTTCTCTGCTGGTCTTTCTTGGCAG GCCACAGGGC CC-3'

A1.1.1 PRIMERS:

FP: **5'-** GCCCCGCGAGGTGCACACTG **-3'** (20mer)

RP: **5'-** GGGCCCTGTGGCCTGCCAAG **-3'** (20mer)

A1.1.2 PROCEDURE:

	Stock concentration	Volume (µL)	Final concentration
Distilled water		16.9	
Buffer	5x	5.0	
dNTP	5 µm	1.0	1 µm
Forward primer	100 nm/µL	0.5	50 nm/assay
Reverse primer	100 nm/µL	0.5	50 nm/ assay
Pm Taq		0.1	
DNA		1.0	

A1.1.3 PCR CONDITIONS:

Stage 1: Denaturation:
94 ^{o}C for 5 minutes (x 1 cycle)
Stage 2: Annealing:
94 ^{o}C for 30 seconds (x 35 cycles)
62 ^{o}C for 30 seconds (x 35 cycles)
72 ^{o}C for 40 seconds (x 35 cycles)
Stage 3: Extension:
72 ^{o}C for 7 minutes (x 1 cycle)

Restriction conditions: MspA1I is incubated at 37 ^{o}C for 4 hours using 0.5 µL of the restriction enzyme per 1.5 µL of PCR product

A1.1.4 GEL:

A 3% Agarose gel (with Ethidium Bromide) is prepared. The PCR products are loaded into the wells and run at a voltage of 160 mV for 45 minutes. View under UV illumination and document the image with the UVITEC transilluminator (Cambridge UK)

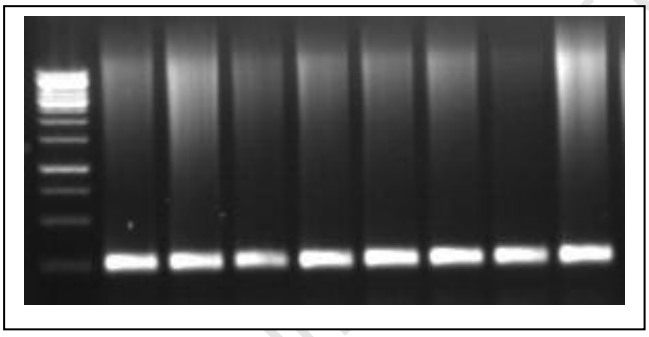

Figure 1 shows PCR products of exon 1 (A19G). Lane 1 is the 1000bp marker ladder; lowest rung equals 250 bp.

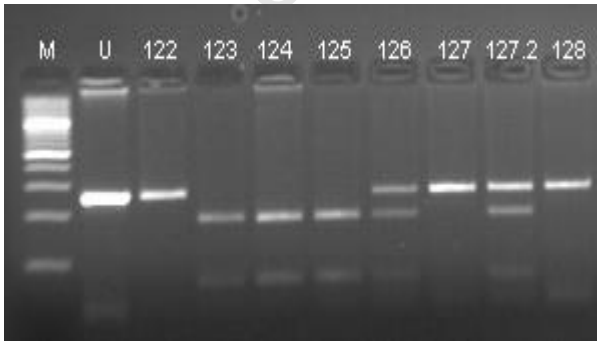

Figure 2: shows RE digest of PCR products of exon 1.
M – Marker (1000bp)
U – Uncut PCR product
Sample 122 – (1 band) 258bp
Samples 123-125 – (2 bands) 186bp and 62bp
Sample 126 – (3 bands) 258bp, 186bp and 62bp

A1.2 PCR ASSAY OF LEP C538T IN EXON 3 OF THE LEP GENE

This polyporphism was first reported by Karvonen *et al* (1998) who found no association between the genotype and obesity phenotypes. It was again reported by Gaukrodger *et al* (2005) who found an association of the T allele with CIMT.

The assay involves producing a fragment of 316 bp with 2 cutting sites at positions 32 and 245 of the fragment for the restriction enzyme TaiII (cuts at 5----A C G T↓----3 or 3----↑T G C A----5). The homozygote genotype (CC) thus has 3 bands: 213bp, 71bp and 32bp fragments, while the heterozygote (CT) has 4 bands: 284bp, 213bp, 71bp and 32bp fragments.

AMPLICON (316)

5′CGACCTGGAGAACCTCCGGGATCTTCTTCACGTGCTGGCCTTCTCTAAGAGCTG
CCACTTGCCCTGGGCCAGTGGCCTGGAGACCTTGGACAGCCTGGGGGGTGTCCTG
GAAGCTTCAGGCTACTCCACAGAGGTGGTGGCCCTGAGCAGGCTGCAGGGGTCTC
TGCAGGACATGCTGTGGCAGCTGGACCTCAGCCCTGGGTGC**TGA**GGCCTTGAAG
GTCACTCTTCCTGCAAGGACTACGTTAAGGGAAGGAACTCTGGCTTCCAGGTATC
TCCAGGATTGAAGAGCATT GCATGGACAC CCCTTATCCA GGAC-3’

A1.2.1 PRIMERS:

FP: **5’-** CGACCTGGAGAACCTCCG- **3**‘(18mer)

RP: **5’** -GTCCTGGATAAGGGGTGT- **3’** (18mer)

A1.2.2 PROCEDURE

	Stock concentration	Volume (x1)	Final concentration
Distilled water		16.9	
Buffer	5x	5.0	
dNTP	5 µm	1.0	1 µm
Forward primer	100 nm/µL	0.5	50 nm/assay
Reverse primer	100 nm/µL	0.5	50 nm/ assay
Pm Taq		0.1	
DNA		1.0	

A1.2.3 PCR CONDITIONS:

Stage 1: Denaturation:
94 ^{o}C for 5 minutes (x 1 cycle)
Stage 2: Annealing:
94 ^{o}C for 30 seconds (x 35 cycles)
53 ^{o}C for 30 seconds (x 35 cycles)
72 ^{o}C for 40 seconds (x 35 cycles)
Stage 3: Extension:
72 ^{o}C for 7 minutes (x 1 cycle)

Restriction conditions: TaiI (MaeII) is incubated at 65 ^{o}C for 4 hours using 0.5 μL of the restriction enzyme per 10.0 μL of PCR product.

A1.2.4 GEL:

A 3% Agarose gel was prepared to run the restriction enzyme digested PCR products. Th products were loaded into the wells and run at 160mv for 45 minutes. This was then viewed under UV illumination and the image documented as shown below.

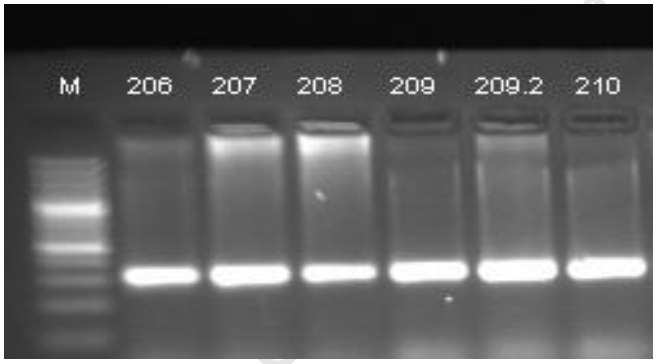

Figure 1: Shows PCR products of exon 3 (C538T). Lane 1 is the marker with 1000bp ladder. 3rd rung from bottom corresponds to 300bp fragments.

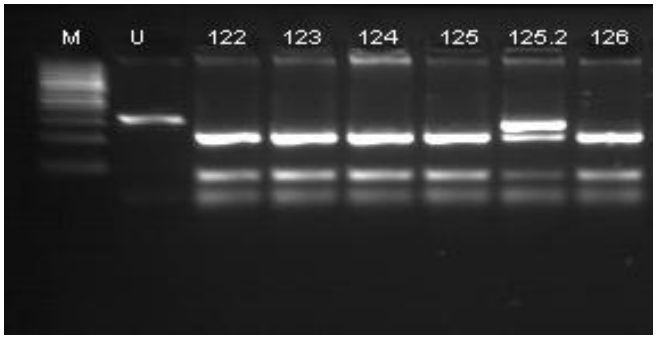

Figure 2: Restriction enzyme digests of PCR products of exon 3.
M – Marker (1000bp)
U – Uncut PCR product 321bp
Samples 122 – 125 (3 bands) 213bp, 71bp, and 32bp
Sample 125.2: (4 bands) 284bp, 213bp, 71bp and 32bp.

A1.3 PCR ASSAY OF LEP C188A IN THE PROMOTER REGION OF THE LEP GENE

A number of studies have reported about this polymorphism and its association with obesity and serum leptin concentrations. Assaying for this SNP involves producing a fragment of 261bp with 1 cutting site for the restriction enzyme AscI. This cut at position 221 produces 2 fragments (221bp and 40bp) and represents the homozygote genotype (CC). The heterozygote (CA) has 3 bands representing 261bp, 221bp and 40 bp. The enzyme cuts at 5'---G G ↓C G C G C C---3' or

3'---C C G C G C ↑G G ---5'.

Amplicon (261)

5'CAACGAGGGCGCAGCCGTATGCCCCAGCCCGCTCCGCGGAGCCCCTCACAGCC
ACCCCCGCCCCGACCGCGCCCCGCGGGCTCGAAGCACCTTCCCAAGGGGCTGGTC
CTTGCGCCATAGTCGCGCCGGAGCCTCTGGAGGGACATCAAGGATTTCTCGCTCC
TACCAGCCACCCCCAAATTTTTGGGAGGTACCCAAGGGTGCGCGCGTGGCTCCTG
GCGCGCCGAGGCCCTCCCTCGAGGCCCCGCGAGGTGCACACT3'

A1.3.1 PRIMERS:

FP: **5'** CAACGAGGGCGCAGCCGTAT **3'** (20mer)
RP: **5'** AGTGTGCACCTCGCGGGGCCT **3'** (20mer)

A1.3.2 PROCEDURE:

	Stock concentration	Volume (x1)	Final concentration
Distilled water		16.9	
Buffer	5x	5.0	
dNTP	5 µm	1.0	1 µm
Forward primer	100 nm/µL	0.5	50 nm/assay
Reverse primer	100 nm/µL	0.5	50 nm/ assay
Pm Taq		0.1	
DNA		1.0	

A1.3.3 PCR CONDITIONS:

Stage 1: Denaturation:
95 ^{o}C for 5 minutes (x 1 cycle)
Stage 2: Annealing:
95 ^{o}C for 30 seconds (x 35 cycles)
62 ^{o}C for 30 seconds (x 35 cycles)
72 ^{o}C for 40 seconds (x 35 cycles)
Stage 3: Extension:
72 ^{o}C for 7 minutes (x 1 cycle)

Restriction conditions: AscI is incubated at 37 ^{o}C for 4 hours using 0.5 µL of the restriction enzyme per 10.0 µL of PCR product.

A1.3.4 GEL:

A 3% Agarose gel was prepared to run the restriction enzyme digested PCR products. Th products were loaded into the wells and run at 160mv for 45 minutes. This was then viewed under UV illumination and the image documented as shown below.

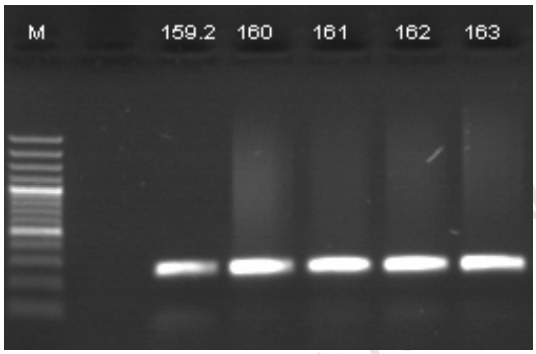

Figure 1: PCR products on a 1% Agarose gel (with Ethidium Bromide). Lane 1 is a 1000bp marker.

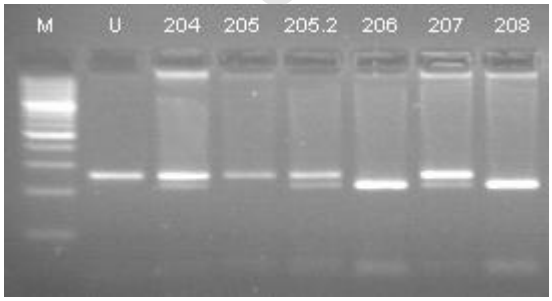

Figure 2: AscI digest of PCR products of C188A.
M – Marker (1000bp)
U – Uncut PCR product (261bp)
204 – 3 bands (261bp, 221bp and 40bp)
205 – 1 band (261bp)
206 – 2bands (221bp and 40bp)

A1.4 PCR ASSAY OF LEP G2548A IN THE PROMOTER REGION OF THE LEP GENE

This SNP appears to be the most studied of the obesity gene, many contradictory reports on its association with obesity and serum leptin levels can be found in the literature. The assay involves producing a small fragment of 109bp with a cut at position 62 with the enzyme HhaI. This cut produces 2 fragments of 62bp and 47bp representing the homozygote genotype (GG). Due to the sizes of these products, only the 62bp fragment is often only seen on the 3% Agarose/Ethidium Bromide gel. Hence, only 1 band is seen in the homozygotes (GG) whereas 2 bands instead of 3 are seen in th heteroygotes (GA). HhaI cuts at 5'---G C G↓ C ----3' or 3'---C↑ G C G ---5'.

AMPLICON:

5'TTTCCTGTAATTTTCCCATGAGAACTATTCTTCTTTTGTTTTGTTTTGCGACAGGG TTGCGCTGATCCTCCCGCCTCAGTCTCCCTAAGTGCTGAGATGTTGCAGGAAG-3'

A1.4.1 PRIMERS

FP: **5'** TTTCCTGTAATTTTCCCGTGAG **3'** (22mer)
RP: **5'** AAAGCAAAGACAGGCATAAAAA **3'** (22mer)

A1.4.2 PROCEDURE:

	Stock concentration	Volume (x1)	Final concentration
Distilled water		16.9	
Buffer	5x	5.0	
dNTP	5 μm	1.0	1 μm
Forward primer	100 nm/μL	0.5	50 nm/assay
Reverse primer	100 nm/μL	0.5	50 nm/ assay
Pm Taq		0.1	
DNA		1.0	

A1.4.3 PCR CONDITION:

Stage 1: Denaturation:
94 ^{o}C for 5 minutes (x 1 cycle)
Stage 2: Annealing:
94 ^{o}C for 30 seconds (x 35 cycles)
48 ^{o}C for 30 seconds (x 35 cycles)
72 ^{o}C for 40 seconds (x 35 cycles)
Stage 3: Extension:
72 ^{o}C for 7 minutes (x 1 cycle)

Restriction conditions: HhaI is incubated at 37 ^{o}C for 4 hours using 0.5 μL of the restriction enzyme per 10.0 μL of PCR product.

A1.4.4 GEL:

A 3% Agarose gel was prepared to run the restriction enzyme digested PCR products. Th products were loaded into the wells and run at 160mv for 45 minutes. This was then viewed under UV illumination and the image documented as shown below.

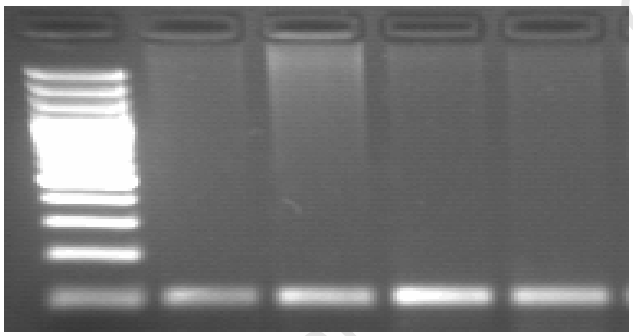

Figure 1: PCR products on a 1% Agarose gel (with Ethidium Bromide). Lane 1 is the marker and the lowest rung is 100bp. PCR product sizes are 109bp

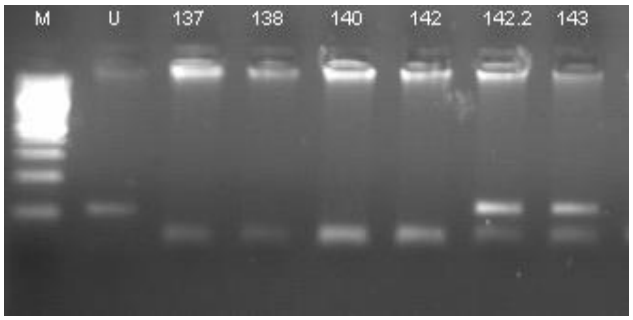

Figure 2: shows HhaI digested bands of PCR products for G2548A.
M – 1000bp marker
U – Uncut PCR product.
137-142: (1 band) 62bp product
142.2 and 143: (2bands) 109bp and 62bp products.

A2: SYMBOLS AND NAMES OF THE 19 COMMON GENES DERIVED FROM TABLE 9.4

Gene symbol	Gene Name
ADCY	Adenylate cyclase
APOB	Apolipoprotein B
ApoE	Apolipoprotein E
AXL	AXL Receptor tyrosine kinase
CAT	Catalase
FOXO1	Forkhead box 01
FYN	FYN oncogene related to SRC, FGR, YES
GIPC1	GIPC PDZ domain containing family, member 1
HSD17B4	Hydroxysteroid (17-beta) dehydrogenase 4
IGF-1	Insulin-like growth factor-1
INSR	Insulin receptor
ITGB1	Integrin beta 1
LDLR	Low-density Lipoprotein Receptor
LPL	Lipoprotein Lipase
LRP1	Low density lipoprotein receptor-related protein 1
PFKM	Phosphofructokinase, muscle
PTPN11	Protein Tyrosine Phosphatase, non-receptor type 11
TIE	Tyrosine kinase with immunoglobulin-like and EGF-like domains
UTRN	Utrophin

A3. PATIENT INFORMATION

INTRODUCTION

You are invited to participate in this study. This information leaflet will help you decide if you would like to participate. Before you agree, you should fully understand what is involved. If you have any questions, which you do not understand, please do not hesitate to discuss this with the study doctor. You should not agree unless you are completely happy about this study, and you are strongly recommended to discuss it with your personal doctor, wherever possible.

WHAT IS THE PURPOSE OF THE STUDY?

You have high blood pressure which can cause damage to the kidneys especially in the presence of some other factors like increased body weight, high levels of lipids (fat) in the blood, etc. We want to see how these factors, together, can cause damage to the kidneys.

WHAT IS THE DURATION OF THE STUDY AND WHAT PROCEDURES WILL BE PERFORMED?

You will be seen once and will undergo full medical examination. Blood and urine tests will be performed. In some cases you may require an oral glucose tolerance test. The test will require you to drink a glucose solution after an overnight fast of about twelve hours during which you are not allowed to take any food but can drink some water. Blood will be taken from you on the morning of the test; at the commencement of the test and at one and two hours from the time of commencement. You may also be asked to come for a kidney biopsy if the results of your urine test indicate you have a moderate-to-severe kidney disease.

HAS THE STUDY RECEIVED ETHICAL APPROVAL?

The Research Ethics Committee of the University of Cape Town has approved the study protocol.

WHAT ARE MY RIGHTS AS A PARTCIPANT IN THE STUDY?

Your participation is entirely voluntary and you can refuse to participate or stop at any time without stating a reason. Your withdrawal will not affect your access to future medical care. The investigator retains the right to withdraw you from the study if it is your best interest.

ARE THERE ANY BENEFITS TO ME?

Early detection of factors that can lead to kidney damage will have definite health benefits to you. If you undergo a kidney biopsy, the exact nature of kidney disease will be identified. There may be no other benefits to you.

MAY THE STUDY PROCEDURES RESULT IN DISCOMFORT OR INCONVINIENCE?

Blood tests may result in a bruise at the puncture site, swelling of the vein, infection, or bleeding. In experienced hands this is highly unlikely and only minor discomfort may occur. (Renal biopsy – refer to separate consent form).

INSURANCE AND FINANCIAL ARRANGEMENTS

All the study doctors are covered by insurance for medical liability and if any study related injury occurs, you have the right to legal action against the study doctor concerned.

You will not be paid to participate but will receive compensation for transport costs for each visit.

SOURCE OF ADDITIONAL INFORMATION

If at any time you have symptoms causing you problems or questions please do not hesitate to contact the investigator at this telephone number. _____

CONFIDENTIALITY

All information during the course of the study is strictly confidential. Data will be reported in scientific journals, but will not include information that identifies you.

It is important that the Research Ethics Committee of the University of Cape Town be able to review records of the study, but only in relation to their regulatory obligations.

A4. INFORMED CONSENT

I hereby confirm that the study doctor (Dr. ----------) has informed me about the nature, conduct, benefits and risks of this study. I have also received, read, and understand the written Patient Information.

I am aware that the results of the study will be anonymously processed into a study report.

I may, at any stage, withdraw my consent and participation without prejudice.

I have had sufficient opportunity to ask questions and declare myself prepared to participate in the study.

Patient's Name: ---------- (Print)

Patient's Signature: ---------- Date: ----------

Study Doctor's Name: ---------- (print)

Study Doctor's Signature: ---------- Date: ----------

Witness Name: ---------- (print)

Witness Signature: ----------

A5. STUDY PROTOCOL

1. SERIAL NUMBER ----------
2. HOSPITAL NUMBER ----------
3. DATE OF INTERVIEW----------
4. DEMOGRAPHIC DATA:

4.1	NAME			
4.2	DOB	YYYY	MMM	DD
4.3	AGE			
4.4	GENDER		M	F
4.5	PHYSICAL ADDRESS			
4.6	TELEPHONE	HOME		
		MOBILE		
4.7	HOME LANGUAGE	XHOSA	ZULU	SOTHO
		TSWANA	OTHER	
4.8	OCCUPATION	UNEMPLOYED	STUDENT	INFORMAL
		FORMAL	PENSIONER	
4.9	ANY RELATIVES IN CAPE TOWN?	Y	N	
4.10	EDUCATIONAL STATUS (SPECIFY)			
4.11	DURATION OF HYPERTENSION (SPECIFY)			
4.12	FAMILY HISTORY OF HYPERTENSION	FATHER	MOTHER	SIBLINGS
4.13	HISTORY OF CVD?	Y	N	
4.14	SMOKING (DURATION) (SPECIFY)			
	SMOKING (No OF STICKS/DAY) (SPECIFY)			
4.15	ALCOHOL PER DAY*	NON-USER	OCCASIONAL	1-2 DRINKS
		3-4 DRINKS	5 OR MORE DRINKS	
4.16	EXERCISE (HRS/WEEK) (SPECIFY)			

*1 Drink equals: One glass of wine / one tot (25ml) of hard liquor (brandy, rum, gin)/ one beer (340 ml)

5. CLINICAL EVALUATION:

5.1	**BLOOD PRESSURE (mmHg)**	SBP	DBP
	RT ARM		
	LT ARM		
	1ST READING		
	2ND READING		
	AVERAGE OF 1ST AND 2ND READING		

5.2	**ANTHROPOMETRY:**	
	WEIGHT (Kg)	
	HEIGHT (m)	
	BMI (Kg/m^2)	
	WC (cm)	
	HIP CIRC. (cm)	
	WHR	

6. LABORATORY EVALUATION:

6.1	OGTT (mmol/L)	TIME 0 min	TIME 60 min	TIME 120 min
6.2	Triglyceride (mmol/L)			
6.3	HDL-cholesterol (mmol/L)			
6.4	LDL-cholesterol (mmol/L)			
6.5	Fibrinogen (mg/dl)			
6.6	CRP			
6.7	Insulin			
6.8	Leptin			
6.9	Adiponectin			
6.10	Creatinine			
6.11	Albumin			
6.12	Urine ACR			
6.13	**GENOTYPE**			
	G2548A	GG	AG	AA
	C188A	CC	CA	AA
	A19G	GG	AG	AA
	C538T	CC	CT	TT

A6: PUBLICATIONS AND CONFERENCE PRESENTATIONS FROM THIS STUDY:

"Association of Microalbuminuria and the Metabolic Syndrome"
Abstract book: South Africa Renal Congress, Cape Town.
March 2006.

"CRP is elevated in Obese Subjects with the Metabolic Syndrome"
Abstract book: South Africa Renal Congress, Cape Town.
March 2006.

"Plasma Leptin and Metabolic Risk Profiles in Hypertensive Black South Africans."
Joint Conference of the Royal college of Physicians and the College of Medicine South Africa, Cape Town.
February 2007.

"The Metabolic syndrome and Early Kidney disease in non-diabetic black Africans"
Abstract book: 2nd International congress on pre-diabetes and the metabolic syndrome, Barcelona, Spain.
April 2007.

"LEP C538T Polymorphism and Obesity related phenotypes: An Association Study in Black Hypertensives."
Abstract book: South African Hypertension Congress, Johannesburg, South Africa.
May 2007.

"A Polymorphism in the 5' UTR Region of the Obesity Gene is associated with Kidney Disease in Black Africans".
Abstract book: South African Renal Society, Durban
July 2008

PUBLICATIONS

"LEP C538T Polymorphism and Obesity related phenotypes: An Association Study in Black Hypertensives." *Cardiovasc J Afr* 2007; 18: (3 suppl) S23-24 (Abstract).

"Microalbuminuria and the metabolic syndrome in non-diabetic black Africans" *Diabetes Vasc Dis Res* 2007;**4**: 365-367.

"Prioritization of candidate disease genes for metabolic syndrome by computational analysis of its defining phenotypes". Physiol Genomics. 2008; 35: 55-64

ARTICLES YET TO BE PUBLISHED

1. Plasma Leptin and Metabolic Risk Profiles in Hypertensive Black South Africans.

2. The LEP A19G polymorphism is associated with markers of early kidney disease in black Africans with Hypertension.